

**Investigations of protein synthesis
dependent long-term potentiation and the
role of dopamine in long-term memory.**

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Ph.D Thesis

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Declaration

In accordance with postgraduate degree regulation 3.8.7 of the University of Edinburgh, I declare that the work described in this document is my own, except where otherwise indicated, and that this thesis was composed by myself.

Colin Michael O' Carroll.

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Abstract.

The aim of this thesis was to explore the temporal persistence of hippocampal long-term potentiation (LTP), with particular reference to the role of dopamine in triggering a protein-synthesis dependent late-phase of LTP (L-LTP). A related aim was to examine the relationship between the induction of L-LTP and the persistence of long-term memory.

Using *in vitro* electrophysiological methods, it was possible to maintain hippocampal slices for prolonged periods. In these experiments, electrical stimulation of two independent input pathways in the CA1, Schaffer-collateral commissural system allowed very stable recordings of extracellular field potentials for > 8 hr. A long-lasting potentiation, or L-LTP, could be induced by 'strong' high frequency tetanisation. Surprisingly, however, this potentiation was not completely blocked by protein synthesis inhibitors (anisomycin or emetine), although the magnitude of the potentiation was reduced in the presence of emetine. Subsequent experiments revealed that temperature affected the magnitude, but not the persistence of L-LTP.

In agreement with a specific prediction of the synaptic tag hypothesis (Frey & Morris, 1997), both a 'strong before weak' and a 'weak before strong' protocol can result in L-LTP on the weakly tetanised input. It is not clear whether this 'strong' tetanic stimulation acts downstream of N-methyl-D-aspartate receptors (NMDARs) or on other neuromodulatory inputs to the hippocampus. Therefore, synaptic tag experiments were conducted in which the 'weakly' stimulated input was preceded by 'strong' tetanic stimulation to another pathway in the presence of the NMDAR antagonist, D-2-amino-5-phosphonovalerate (APV). The ability of 'strong' stimulation to rescue LTP on the weakly tetanised input was dependent upon activation of the NMDA receptor during the 'strong' tetanus. Synergistic activity between both NMDA and dopaminergic receptors may be required to induce persistent L-LTP. Experiments demonstrated that the activation of dopamine receptors is important both during and after the tetanic stimulus.

Parallel behavioural studies were conducted using a repetitive one-trial learning paradigm called the "delayed-match-to-place" in the watermaze. Intra-hippocampal infusions in rats, of SCH23390 were found to inhibit performance, only at long memory intervals, and not short-term intervals. Both the electrophysiological and behavioural data are discussed in relation to the synaptic tag hypothesis.

Chapter 1. Introduction.

1.1 Theoretical background.

The twin aims of this thesis were (1) to explore the temporal persistence of hippocampal long-term potentiation (LTP), with particular reference to the role of dopamine in triggering a protein-synthesis dependent late-phase of LTP (L-LTP); and (2) to examine the relationship between the induction of L-LTP and the persistence of long-term memory.

The phenomenon of memory has fascinated psychologists and neuroscientists; but also writers, philosophers, painters, and musicians. In ancient Greece, memory was held in the highest esteem and rigorously trained. For the Greeks, “*Mnemosyne, the goddess of memory, was also the goddess of wisdom, the mother of the muses...and therefore...the progenitor of all the arts and sciences*”, (Casey, 1987) while the Romans placed memory at the heart of all teaching, learning and thought (Samuel, 1994).

A vast body of evidence has emerged focussing on the role that protein synthesis plays in the storage of long-term memory (for a review see Davis and Squire, 1984). Considerable insight into the possible biological mechanism by which information is stored in the brain arose with the discovery of the phenomenon of long-term potentiation in an area of the brain that is involved in memory, namely the hippocampus (Bliss and Lømo, 1973a). As for memory, long-term potentiation has been found to be dependent on protein synthesis for its long-term persistence (Krug et al., 1984; Stanton and Sarvey, 1984; Frey et al., 1988).

The hippocampus is a critical brain structure that is involved in memory. Long-term potentiation is induced at hippocampal synapses, and in order to maintain the long-term change, protein synthesis must occur. The mechanism by which proteins are able to selectively strengthen individual synapses is controversial, however, one possible mechanism has been proposed, and experimentally tested, by Frey and Morris, (1997). The ‘synaptic tag’ hypothesis provides the conceptual backdrop to all of the experiments discussed in this thesis. We are only beginning to understand how both long-term memory and LTP, may be modulated by non-glutamatergic inputs to the hippocampus. The dopaminergic system was chosen as a possible candidate in these regulatory processes.

1.1.1 Memory loss due to hippocampal damage.

Even in our own daily lives we can hardly deny the pervasive role that memory plays in constructing our sense of ‘self’. Occasions that highlight the importance of memory arise when it fails us. It of no coincidence therefore, that much of our current understanding of memory comes from cases where profound memory problems arise due to damage of the brain. Historically, the most influential case that highlights this point is that of patient H.M. (Scoville and Milner, 1957). Patient H.M. underwent neurosurgery in an attempt to cure severe epilepsy. The surgery involved a bilateral resection of the medial temporal lobe, which includes structures such as the hippocampal formation. As a result of this, H.M. lost the ability to remember facts and events in his past (retrograde amnesia) and the ability to acquire new memories (anterograde amnesia), (Corkin, 1965; Milner, 1965; Milner et al., 1968; Corkin, 1984). Focus centred on the role that the hippocampus plays in memory because the severity of the amnesia was related to the size of hippocampal tissue removed (Milner, 1974); for a recent review see Corkin, (2002).

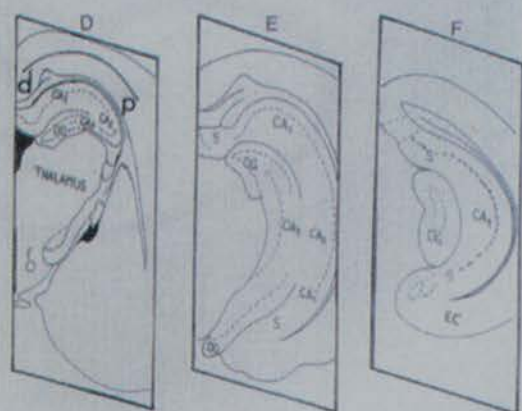
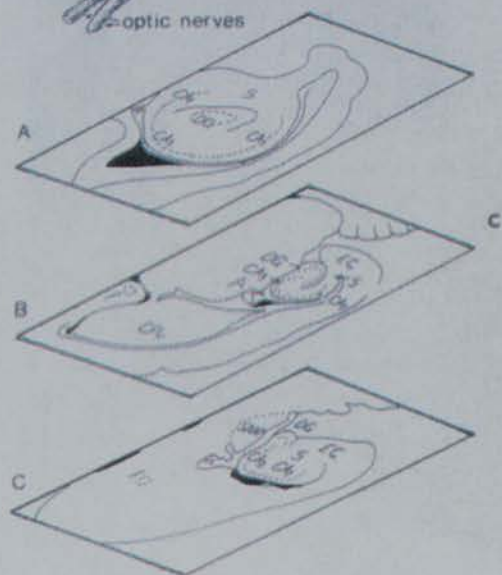
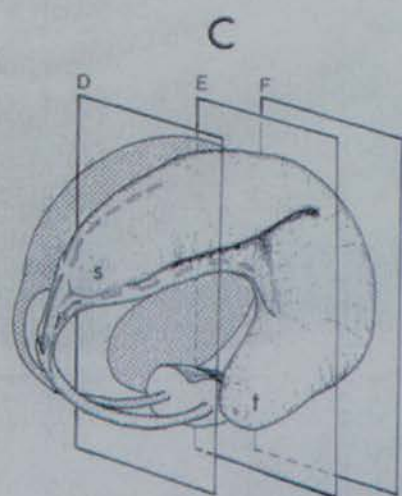
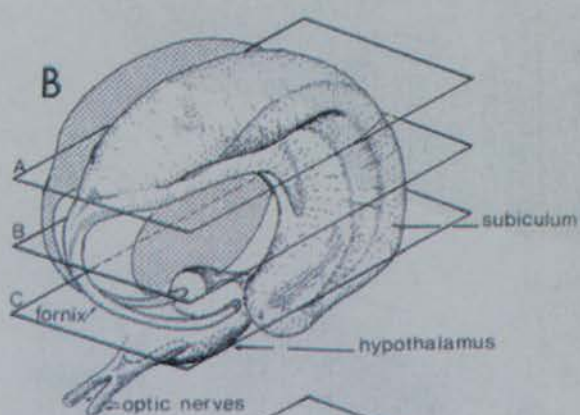
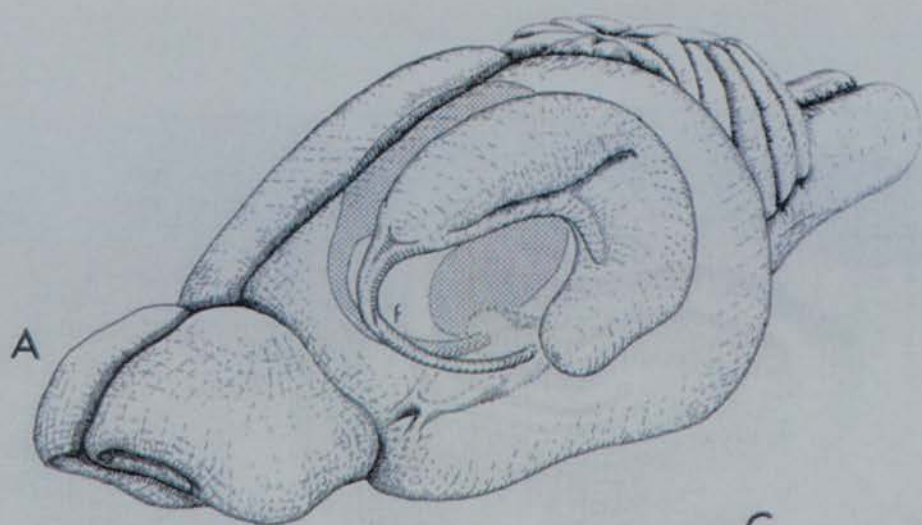
1.2 The hippocampus.

The rat hippocampal formation is located within the forebrain and forms part of the temporal lobe of the cerebral cortex (see Figure 1.1). The position of the hippocampus within the temporal lobe is such that it lies beneath the corpus callosum and stretches caudally from the septum, and latero-ventrally around the thalamus. The hippocampal formation is generally considered to consist of the hippocampus proper, i.e. Ammon’s horn (Cornu Ammonis, CA), the dentate gyrus (fascia dentata), the subicular complex, and the entorhinal cortex. The hippocampus proper is divided into three subfields on the basis of the size of the cells and distribution, regions CA1, CA2 and CA3. Pyramidal cells in CA3 receive a prominent mossy fibre input from the dentate gyrus, which is not present in CA1. The CA2 region is less clearly defined, and its very existence has sometimes been debated. A similar laminar organisation is seen in all layers of the hippocampus proper. The pyramidal cell layer is most prominent, and deep to this layer is a relatively cell-free region called the stratum oriens. Above the pyramidal cell layer lies the stratum radiatum in which CA3 to CA1 Schaffer collateral connections and CA3 associational connections are located. The most superficial layer is known as the stratum lacunosum-moleculare, in which perforant path fibres from the entorhinal cortex terminate.

Figure 1.1. The rat hippocampus.

Schematic drawing illustrating the position of the hippocampus in the rat brain (a) and the relative position of the different components as observed in horizontal (b) and coronal (c) sections. Abbreviations: f, fornix; EC, entorhinal cortex; DG, dentate gyrus; S, subiculum; s, septal pole; t, temporal pole.

From (Amaral, 1989).



1.2.1 Intrinsic pathways of the hippocampus.

Many important aspects of hippocampal physiology, including LTP, can be studied in the *in vitro* hippocampal slice. The salient features of the intrinsic connectivity of the hippocampus are often characterised as a 'tri-synaptic loop'. The projections within this loop are almost exclusively unidirectional. The circuit starts with perforant path afferents which synapse onto granule cells of the dentate gyrus. The axons of these granule cells form the mossy fibre pathway, which makes *en passant* synapses with the dendrites of the CA3 pyramidal cells. The axons of the CA3 pyramidal cells form the Schaffer collateral pathway which projects to the CA1 pyramidal cells. In addition to forming the Schaffer collaterals, the axons of CA3 pyramidal cells divide and give rise to associational projections to other CA3 pyramidal cells, as well as commissural projections to CA1 and CA3 pyramidal cells in the contralateral hippocampus. The pyramidal cells of CA1 project back to the entorhinal cortex, and indirectly via the subiculum.

On the basis of electrophysiological and neuroanatomical studies, it was proposed that the major excitatory pathways of the tri-synaptic loop run almost exclusively in a direction perpendicular to the septotemporal axis of the hippocampus, with a highly restricted lateral spread (Andersen et al., 1969; Andersen et al., 1971). According to this view, the hippocampus is composed of a large number of semi-autonomous transverse slices, a structure reminiscent of the columnar organisation of the neocortex. However, Amaral (1989), has challenged this notion on the basis of new neuroanatomical evidence suggesting that with the exception of the mossy fibre pathway, many of the intrinsic hippocampal connections are as extensive in the septotemporal direction as in the transverse direction. Furthermore, different projection systems have distinct and highly topographically organised patterns of organisation in the septotemporal axis. Hence, the lamellar hypothesis in its original form may be an oversimplification. As mentioned above, many important aspects of hippocampal physiology, including LTP, can be studied in the *in vitro* hippocampal slice, a convenient experimental preparation in which transverse hippocampal sections of ~ 400 μm in thickness are maintained by bath perfusion with aCSF. Proponents of the lamellar hypothesis question whether slice preparations would work if a large part of the intrinsic circuitry were discontinuous as implied by Amaral (1989).

1.2.2 Extrinsic input pathways to the hippocampus.

The entorhinal cortex provides the major sensory input to the hippocampus. The entorhinal cortex receives highly processed multimodal information from several sensory regions of association cortex, as well as a direct projection from the olfactory cortex. This information is conveyed to the hippocampus by the perforant pathway. This projection arises in layer II and III of the entorhinal cortex, and forms the dense “angular bundle” upon entering the hippocampus. The perforant path can be subdivided into medial and lateral components, which terminate in the middle one-third and outer one-third of the dentate molecular layer respectively.

1.2.3 Neuromodulatory inputs to the hippocampus.

In addition to the glutamatergic input (i.e. the perforant path input), the hippocampus receives a number of projections from sub-cortical structures. Some of these projections may have important modulatory roles on the processing of information within the hippocampus. For example, a serotoninegic projection arises in the raphe nucleus and projects to the hippocampus via the fornix. A noradrenergic input is provided via the locus coeruleus. One pathway that is of particular relevance to the experiments of this thesis is the dopaminergic projection to the hippocampus from the ventral tegmental area (Carter and Fibiger, 1977; Swanson, 1982; Gasbarri et al., 1994a; Gasbarri et al., 1994b). Another important neuromodulatory input is that made by the basolateral nucleus of the amygdala, which projects to the ventral part of the hippocampus.

1.2.4 Projections from the hippocampus.

The subiculum provides the major output from the hippocampus, and gives rise to a large projection back to the entorhinal cortex, as well as to a number of neo-cortical areas. Further, the subiculum projects to a number of subcortical areas, including the septal complex, the mammillary bodies, the nucleus accumbens and the thalamus. Neurons of the CA1 also project directly to a number of cortical areas, amygdala and lateral septum, along with the projections via the subiculum.

1.3 Short-term and long-term memory.

Memory is not a unitary process of information storage, but rather consists of multiple processes, each with different time courses. It is therefore likely that information retained for a relatively short or for a long period of time will be dependent on different processes taking place during the initial memory ‘trace’ formation and on processes taking place during consolidation and possibly retrieval. Although the precise role that the hippocampus plays in memory is still intensely debated, it is generally accepted that the hippocampus is involved in declarative memory, i.e. the explicit memory of general facts and everyday life events (Squire, 1989).

1.3.1 Different ‘types’ of memory.

Considering memory, it is evident that this is not a single unitary phenomenon, but rather is composed of several different components, which are mediated by separate brain systems. Traditionally one can differentiate between declarative memory (explicit memory of general facts and everyday life events) and non-declarative memory (procedural memory for skills and the ability to respond to stimuli through practice). This distinction has arisen from studies of amnesic patients, and suggests how different brain structures are involved in different types of memory (Squire, 1992). Declarative memory systems are more than likely to be more developed in mammals, whereas invertebrates and non-mammalian species may not possess declarative memory. A possible exception may arise when considering birds, as recent evidence suggests that some species of birds such as Scrub Jays, seem to possess some episodic-like memory abilities (Clayton and Dickinson, 1998). However, similar synaptic and molecular mechanisms may be at work in both declarative and non-declarative. The long-term storage and consolidation of declarative memories, which involve ‘higher’ cortical systems (and interactions among different neuronal networks), is likely to involve a level of complexity not realised in systems which possess the neural machinery adapted for non-declarative memory.

1.3.2 Distinguishing between short-term and long-term memory.

A widely accepted idea is that memory storage in the mammalian brain can be divided into at least two temporally distinct phases: short-term memory (STM), lasting minutes to hours, and long-term memory (LTM), which can persist for days, weeks, or even years. However, it is

important to ask some critical questions when such distinctions are made. It is in some ways quite arbitrary what is meant by 'long-term'. Psychologists studying the memory of their subjects may consider long-term memory as referring to events remembered which occurred years in the past. For the behaviourist working with a rat in tasks that require retention of information acquired during training, ~12 hr can be taken to be long-term memory. On the other hand, for a physiologist studying the changes occurring in populations of cells, or even individual neurons, long-term may mean anything lasting a couple of hours.

1.3.3. Cellular and systems consolidation.

It is important to make a distinction between what has been termed 'cellular consolidation' and 'systems consolidation' (Dudai, 1996). Cellular consolidation refers to those processes that take place within individual neurons undergoing activity-dependent alterations in synaptic plasticity. These processes will involve changes both locally at individual synapses, and more diffusely in the neuronal cell bodies. These processes are likely to take place during, or shortly after learning takes place. Systems consolidation on the other hand, involves changes that take place in the overall circuits that are engaged during the learning experience, and may involve reorganisation of the circuits that encode the traces in a number of different regions of the brain. This is likely to take place over a longer time frame than that associated with cellular consolidation. As individual synapses grow and change, the memory trace encoded may not solely be dependent on these exact synapses. As systems level consolidation occurs, the 'location' of any particular memory trace may be 'distributed' over a wide network of synapses in a number of different brain areas. This process is generally believed to transform a labile or 'weak' memory, into something more persistent, or 'stronger'.

1.3.4. Consolidation of long-term memory.

As consolidation takes place, the information stored is thought to become less susceptible to disruption, as may arise for example, when the brain is damaged. Clinical observations led Ribot (1882), to formulate the "*Loi de regression*", which proposed that events experienced immediately before a brain injury were the most likely to be forgotten. Events in the distant past appeared to be relatively spared. In relation to patient H.M., certain short-term memory functions are intact (due to their dependence on extra-hippocampal structures), but he has severe problems in many aspects of long-term memory (Wickelgren, 1968). This observation along with the fact that H.M. was not able to form new memories, but could recollect some

events prior to his surgery led to the idea that the ‘location’ of well established remote memories is not within the hippocampus. Rather, the hippocampus is an important structure involved in the encoding of memories that ultimately reside within neo-cortical areas (Squire and Zola-Morgan, 1991; Zola-Morgan and Squire, 1991). It is not the case that the hippocampus is involved only in short-term and not long-term memory formation, it may be involved in both. This again reflects the idea of ‘systems consolidation’ processes that may involve many brain areas in addition to the hippocampus. The exact nature of the role of the hippocampal formation in consolidation, and the interaction with cortical areas, is beyond the immediate scope of the experiments of this thesis. However, this point concerning consolidation of long-term memory, and interactions between the hippocampus and other structures, will be addressed again later. For a relevant perspective on this, see McGaugh, (2000).

1.3.5. Long-term memory requires protein synthesis.

Katz and Halstead (1950) were one of the first to propose that memory involved protein molecules. Protein synthesis inhibitors applied either before, or during training, have no effect on either the initial acquisition of certain tasks or short-term memory, but disrupt memory that is acquired during training if animals are tested at a long memory interval (Flexner et al., 1963). These reports provide the historical backdrop to much subsequent research aimed at asking what processes may be involved in long-term storage. The general consensus from research on a variety of species is that protein synthesis during, or shortly after, learning is required for the formation of long-term memory. The initial acquisition and retention of short-term memory is independent of protein synthesis. This effect is due to the inhibitors interfering with processes involved in the storage but not the retrieval of long-term memory. For further review see Davis and Squire, (1984).

One alternative explanation is that the amnesic effect of these drugs may not be due to the effects on the synthesis of new proteins, rather, they may cause a run-down of constitutive brain proteins. However, intracerebral injection of the inhibitor acetoxycycloheximide 18 hr before training (which results in a high level of protein synthesis inhibition many hours before training, but subsequently drops below an effective dose for a significant block at the time of training) has no effect on long-term memory (Barondes and Cohen, 1967). Similar results have been found more recently with injections of anisomycin 24 hr prior to both auditory and contextual fear conditioning (Schafe et al., 1999). These experiments both involved

vertebrates. Invertebrates, such as the marine mollusc *Aplysia*, also show the same dependence on the synthesis of new proteins for long-term memory (Montarolo et al., 1986; Schacher et al., 1988).

1.3.6 Multiple periods of protein synthesis.

Most studies (including those described above) have emphasised the role of a single period of protein synthesis initiated at the time of training or shortly thereafter. However, other studies suggest that there are two or more sensitive periods during which protein synthesis inhibitors may exert their effect (Grecksch and Matthies, 1980; Freeman et al., 1995; Chew et al., 1996; Bourtchouladze et al., 1998; Tiunova et al., 2000). Freeman et al., (1995), report two distinct time windows for the amnesic effect of the protein synthesis inhibitor anisomycin in chicks trained on a passive avoidance task. Contradictory findings exist as Abel et al., (1997) found that anisomycin was only effective at producing long-term memory deficits when injections were made 30 min before training in mice trained in a contextual fear conditioning paradigm. No effect was observed when injections were made 1, 3, 4, 6, 8 or 23.5 hr after training.

Bourtchouladze et al., (1998) also report that there is a pattern of at least two different periods in which inhibitors produce long-term memory deficits. This study suggests that the ‘type’ of training the animals were given may determine the number of sensitive periods to inhibitors. Using contextual fear conditioning (for which the long-term retention of the association between the context, the CS, and the painful stimulus, the US, has been reported to depend on protein synthesis, (Abel et al., 1997; Schafe et al., 1999), animals were trained with either one pairing of the CS/US (‘weak’ training), or three pairings (‘strong’ training). With the ‘weak’ protocol, mice injected with anisomycin immediately after training showed normal performance when tested 1 hr later, but no long-term retention at a number of intervals from 3–24 hr. When animals were given a ‘strong’ training protocol, anisomycin was effective at blocking long-term retention when administered immediately after training but not when administered at later times. However, with the ‘weak’ protocol, when injections were made 4 hr after training significant effects on retention tested 24 hr later were found, but not when injections were made 1, 6, 8 or 23.5 hr after training. This dissociation raises the possibility that the type of training influences the number of protein synthesis sensitive periods that will follow. This may be due to a recruitment of a number of different signal transduction pathways in response to ‘strong’ training, in contrast to the ‘weak’ protocol. The ‘weak’ training was, nonetheless sufficient to produce robust long-term memory at 24 hr, such that,

although a common end-point may be reached, there are numerous paths to get there. However, the experiments of Abel et al., (1997) only used one exposure to the conditioning chamber (i.e. ‘weak’ training according to the distinction made by Bourtchouladze et al., 1998), so why they only find one sensitive period for anisomycin is unclear.

1.3.7 The ‘trial-spacing’ effect.

When animals are trained on a variety of tasks, it has been generally found that more robust memories are produced when the learning trials are spaced apart in time rather than being massed. Tully et al., (1994) found that *Drosophila* trained with a temporally spaced protocol displayed improved long-term memory compared with flies trained with a massed protocol. The facilitatory effect that spaced training has on long-term retention is sensitive to protein synthesis inhibition. In mice lacking both α and δ isoforms of CREB, spaced training was also found to selectively rescue long-term memory deficits (Kogan et al., 1997), and rats trained in a fear-potentiated startle paradigm show enhanced long-term memory following spaced training (Josselyn et al., 2001). One explanation for this spaced-trial phenomenon is that, with repetitive training at short inter-trial intervals the processing of information from a trial is attenuated whilst information from a previous trial is still being processed, suggesting that some minimal delay is required to form a stable representation (Spear and Riccio, 1994).

Taken together, the common theme that emerges from this research is that protein synthesis is required for the storage of long-term memories. The critical period in which synthesis is required is usually during, or shortly after the episode that triggers processes involved in long-term storage. However, both the time and number of critical periods may be influenced by the nature of the inducing stimulus (i.e., the training schedule), or the temporal order and time between successive learning episodes.

1.4 Modulation of memory.

1.4.1 Neuromodulation of memory formation.

Over the years, experimental work has focussed attention on the concept of modulation of memory storage. For reviews see (Cahill and McGaugh, 1996; McGaugh, 2000; Cahill et al., 2001; McGaugh, 2002). In recent years, this area of memory research has begun to generate

new ideas that point to the role that interactions between different brain regions play in the long-term storage of memory. There is some historical precedent to this work, as Goddard, (1964) reported that electrical stimulation of the amygdala produced retrograde amnesia. The experiments reported in Chapter 7 focus on the modulatory role of dopaminergic input to the hippocampus in long-term memory. Considerable evidence has accumulated suggesting that the storage of memories can be influenced by a number of modulatory systems.

Research has focussed on how particular stress hormones, such as epinephrine and cortisol (corticosterone in the rat) are released by emotionally arousing stimuli, and how these hormones modulate the subsequent storage of certain types of memories. Epinephrine, corticosterone, and drugs that activate adrenergic and glucocorticoid (type II) receptors enhance memory in a number of tasks (Gold and van Buskirk, 1975; Sandi and Rose, 1994; Lupien and McEwen, 1997). Activation of the amygdala is critical for mediating the influences of epinephrine and glucocorticoids, because amygdala lesions block the modulatory effects that these agents exert on consolidation. Systemic post-training administration of the synthetic glucocorticoid dexamethasone, enhanced memory for inhibitory avoidance, and this enhancement was blocked in animals with excitotoxic lesions of the basolateral nucleus of the amygdala (BLA) but not of the central nucleus (CEA) (Roosendaal and McGaugh, 1996). Activation of β -adrenergic receptors within the amygdala appears to be central to this modulatory effect, as infusions of β -adrenergic receptor antagonists into the amygdala after training block the facilitatory effects normally produced by epinephrine. Infusions of β -adrenergic receptor agonists also enhance memory (Liang et al., 1986).

1.4.2 Neuromodulatory input to the hippocampus from the basolateral amygdala.

Some of the earliest evidence that the amygdala plays a role in regulating memory consolidation comes from work by Gallagher et al., (1977) who demonstrated that β -adrenergic-receptor antagonists infused into the amygdala impaired retention 24 hr later provided infusions were made immediately but not 6 hr after inhibitory avoidance training. Many subsequent studies have shown that noradrenaline produces dose-dependent, and time-dependent enhancement of memory consolidation when infused into the amygdala shortly after either inhibitory avoidance training (Liang et al., 1986) or performance of a watermaze task (Hatfield and McGaugh, 1999). The amygdaloid complex consists of a number of subnuclei; the basolateral amygdaloid nucleus (BLA), the basomedial amygdaloid nucleus

(BMA), the central amygdaloid nucleus (CeA), and the medial amygdaloid nucleus (MeA) (Amaral et al., 1992). Neural projections from the amygdala to the dentate gyrus of the hippocampus have been demonstrated by several anatomical and physiological studies (Racine et al., 1983; Thomas et al., 1984; Aggleton, 1985, 1986; Saunders et al., 1988; Ikegaya et al., 1996b).

The BLA projects to many brain regions including various cortical regions, the hippocampus, basal forebrain, the nucleus accumbens, and the striatum (Pitkanen, 2000). Experiments have shown that the amygdala modulates memories that are known to be hippocampal dependent (at least for the initial formation of the memory trace). Packard et al., (1994) made the suggestion that, if the amygdala modulates memory in the hippocampus, stimulation of the amygdala should influence the formation of memory traces that involve this structure, e.g. spatial memory in the watermaze. Additionally, after modulation by amygdala stimulation, inactivating the amygdala during retention testing might not disrupt this memory. Amphetamine was infused into the amygdala, hippocampus, or caudate nucleus, immediately after rats were trained in either a spatial watermaze task, or a non-spatial visually cued watermaze task. Whereas amphetamine infused into the caudate selectively enhanced memory on the visually cued task, hippocampal infusions enhanced performance on the spatial task. However, amygdala infusions affected both tasks equally. Furthermore, inactivation of the amygdala (with lidocaine infusions) just before retention testing did not block the expression of the enhanced memory of either the cued or spatial task. This indicates that the 'locus' of the memory is not within the amygdala, but rather, the amygdala can modulate memories that are processed elsewhere. The finding that infusion of β -adrenergic-receptor antagonists into the BLA block the memory enhancing effects of a glucocorticoid-receptor agonists administered into the hippocampus after training strengthens the suggestion that the BLA plays an important role in modulating hippocampal function (Roosendaal et al., 1999).

1.4.3 Modulation of short-term vs. long-term memory formation.

All of the above studies involve post-training manipulations to assess the effect that these may have on memory consolidation. Comparatively less is known about how pre-training manipulations involving these various neuromodulatory pathways may affect the subsequent storage of memories.

One reason may be that many of the pretraining manipulations discussed above could act on processes involved in the initial acquisition rather than the storage of the information in memory. However, one example of a pre-training manipulation that appears to affect long-term and not short-term memory was reported in an experiment by Josselyn et al., (2001). Earlier, evidence was presented that spaced training trials produce more robust long-lasting memories than massed training. Later sections will focus on the role that CREB plays in the synthesis of proteins necessary for the formation of long-term memory. Josselyn et al., (2001) increased CREB levels in the BLA via viral vector-mediated gene transfer. They report that rats trained in a fear-potentiated startle paradigm on a massed training schedule (that normally produces poor long-term memory) showed enhanced long-term memory measured 48 hr after training if they had received injections of the CREB virus 3 days before the initial training episode. Levels of CREB over-expression were significantly elevated 3 days after the injection but not at 14 days. A second retention test was performed 14 days after the injection. Rats that had been injected 14 days before training i.e. trained at a time when transgene expression was not elevated, did not show enhanced long-term memory when tested 14 days after training, whereas the 3 day group still showed robust memory when tested in this second 14 day retention test. As CREB levels in this 3 day injected group would have been low at the time of testing this indicates that the CREB over-expression affected encoding rather than retrieval.

1.5 Long-term potentiation

Long-term potentiation in the hippocampus is the most extensively studied form of activity-dependent synaptic plasticity in the mammalian brain. Although LTP occurs in many pathways in the brain, most research has focussed on the properties of hippocampal LTP, particularly NMDA receptor dependent LTP. Another focus has been on the possible links between LTP and specific types of memory. Key aspects of the review that follows are the signalling mechanisms involved in different 'phases' of LTP. The role of protein synthesis in long-term memory has been introduced already and a similar dependence on protein synthesis for a particular 'phase' of LTP, called late-LTP (L-LTP) has also been reported.

1.5.1 Searching for the ‘engram’.

Ramon y Cajal, (1909) was the first to suggest that information could be processed by changes in the connections between neurons, which Sherrington, (1947) was later to term ‘synapses’. Hebb, (1949) subsequently proposed a set of circumstances in which these changes could occur. He postulated that information could be stored by changes in the strength of the connections between simultaneously active nerve cells. The first connections in the mammalian brain in which these changes were observed, are those made by entorhinal perforant path fibres onto dentate granule cells in the hippocampus (Bliss and Lømo, 1973a). Brief, high-frequency stimulation of this pathway elicited a long-lasting enhancement of synaptic transmission. This phenomenon was later termed ‘long-term potentiation’ or LTP. The phenomenon of LTP has been the focus of intense research ever since these original theoretical and experimental observations. The effect can also be seen in slices of the hippocampus maintained *in vitro* (Schwartzkroin and Wester, 1975).

1.5.2 What is LTP?

“LTP is expressed as a persistent increase in the size of the synaptic component of the evoked response, recorded from individual cells or from populations of neurons. It can be induced in a number of ways, most conveniently, by delivering a tetanus (typically a train of 50-100 stimuli at 100Hz or more) to the pathway of interest” (Bliss and Collingridge, 1993). LTP is not a unitary phenomenon but rather can be divided into a number of processes including induction, expression and persistence. Each of these processes may involve different mechanisms.

1.6 LTP and learning.

Some of the first experimental evidence that suggested that the phenomenon of LTP may play an important role in memory comes from research which showed that the persistence of LTP was correlated with the rate of learning and/or the amount of information retained over time. (Barnes, 1979; Barnes and Mc Naughton, 1985). Another spatial task is the ‘open-field’ watermaze. This task has been shown to be very sensitive to hippocampal dysfunction (Morris et al., 1982). It has been suggested that the requirement of having to learn the complex relationships of extra maze visual cues is the essential feature of this task that makes it sensitive to disruption of the hippocampus (Morris, 1984, 1990).

1.6.1 Blockade of the NMDA receptor and effects on spatial learning.

A critical and highly influential experiment was that of Morris et al., (1986), who found that pharmacological blockade of the NMDA receptor with the selective antagonist AP5, blocked spatial but not visual discrimination learning in rats. The dose-response profile at which AP5 blocks learning was subsequently found to be similar to that which blocks LTP (Davis et al., 1992). Subsequently it was reported that application of AP5 after training in the watermaze did not affect the retrieval of previously learned spatial information (Morris, 1989). These results (Morris et al., 1986; Morris, 1989), suggest that NMDA receptor activation is necessary for the acquisition, but not recall of spatial information. This is reflected by experiments showing that AP5 affects the induction, but not expression of LTP (Davies and Collingridge, 1989).

1.6.2 Sensorimotor side effects produced by NMDA receptor antagonism.

A caveat is that the NMDA receptor-dependent blockade of learning has been suggested to be due to sensorimotor side effects associated with this drug (Cain et al., 1996; Saucier et al., 1996). The experiments reported above (Morris et al., 1986; Morris, 1989) used intraventricular injections to deliver the drug to the brain. The behavioural deficit associated with drug infusion was later found to be ameliorated by pretraining which also prevented the drug induced side effects (Saucier and Cain, 1995), although LTP was still blocked. Other studies showed that if animals are given pre-training that is non-spatial in nature, the AP5 induced deficit is present. If the pre-training is spatial in nature, then the animals no longer show the deficit associated with NMDA blockade and perform similar to non-drug treated control animals (Bannerman et al., 1995).

A more convincing way to examine whether NMDA receptors are important for learning and memory, and that blockade of these receptors affects processes primarily associated with encoding, is by examining whether there is a delay-dependent effect independent of any side-effects. Steele and Morris, (1999), trained rats in a delayed-match-to-place version of the watermaze. In this task, rats are trained to find a novel platform position each day (the platform remaining in a constant position within each day). Hence, performance on trial 2 represents an opportunity to match-to-place by the rat remembering the location of the platform on trial 1 after variable delays. The findings indicated that rats infused with D,L-AP5 via chronic i.c.v. mini-pumps or acute intra-hippocampal infusions were unimpaired at the shortest delay of 15s between trial 1 and 2. However, with either a 20min or a 2hr interval,

the AP5 treated rats showed no memory of the platform position. Thus, some hippocampal NMDA receptor-dependent mechanism is essential for remembering spatial information for 20 min or longer. It is tempting to link this to LTP.

1.6.3 Genetic evidence for a link between LTP, learning and memory.

In recent years, new molecular biological approaches to the analysis of both LTP and memory have been introduced. Throughout the present discussion of experiments related to both memory and LTP, studies are cited whereby transgenic animals are used as a 'tool' in order to dissect the involvement of different signalling pathways in LTP and memory. Studies of this kind usually follow a regime whereby the gene is deleted, this is confirmed by various biochemical assays and then there is extensive phenotypic characterisation with anatomical, electrophysiological and behavioural analyses. This growing body of evidence is important but not of direct relevance to this thesis. For a discussion of the possible link between LTP and learning, see Martin et al., (2000); Martin and Morris, (2002); for a discussion of the effect of genetic background of the mice used in transgenic studies see Gerlai, (1996, 2001), Gerlai et al., (2002); and Nguyen et al., (2000) for an example of how LTP varies between different inbred mouse strains. Only a brief survey follows here.

Some of the early pioneering work of this kind revealed that there are correlations between deficits in hippocampal LTP and learning (Grant et al., 1992; Silva et al., 1992a; Silva et al., 1992b). Silva et al., (1992a, 1992b) generated mice lacking the α -subunit of CaMKII which showed a spatial learning deficit in the watermaze and a deficit in LTP *in vitro*. Normal synaptic transmission was unaffected in these animals, although they did show some initial impairment in a visual-cue version of the watermaze.

Many subsequent studies whereby homologous recombination is used to delete genes in all cells of the body for the lifetime of the animal have been conducted. This makes a clear understanding of the causal relationship between the gene of interest and learning problematic. Mice deficient in the γ -subunit of PKC show a mild impairment in spatial learning, but a profound impairment in CA1 LTP (Abeliovich et al., 1993a; Abeliovich et al., 1993b). It was subsequently discovered that the deficit in LTP could be 'rescued' by preceding the LTP tetanus with priming stimulation that consisted of 900 pulses at 1Hz. Another study found that mice deficient in *Thy-1*, a neuronal cell adhesion molecule, performed normally in the watermaze, even though *in vivo* (under anaesthesia) LTP recorded

in the dentate gyrus was disrupted (Nosten-Bertrand et al., 1996). However, Errington et al., (1997) later found that dentate LTP could be elicited in freely moving *Thy-1* mutant mice.

Considerable questions were raised about these ‘early’ transgenic studies as none of the mutations were restricted to specific brain regions, and the genes are missing throughout development of the animal. Some critical proteins may be important in a number of processes during development, such that deletion of these molecules may result in embryonic or perinatal lethality. Even if the animal survives, the fact that the gene of interest will be missing from the entire brain poses logical problems in attributing a learning deficit to the absence of the gene from the hippocampus. It has been reported that *fyn* mutant mice (which show both LTP and learning deficits, (Grant et al., 1992) tend to spend a lot of time floating in the watermaze, which gives rise to long escape latencies. Lightly touching the hind feet of the mice at the start of a trial was found to improve swimming, such that the learning deficit was no longer present (Huerta et al., 1996). However, recent advances with both tissue and regional specific ‘knock-outs’ using the *Cre-lox P* system (Tsien et al., 1996), and the use of inducible promoters (Furth et al., 1994; Kistner et al., 1996; Mayford et al., 1996b), has taken this field forward considerably.

1.7 The ‘classical’ properties of LTP in the hippocampus.

Many people have suggested that LTP is an ideal candidate for the way in which synaptic changes may encode memories due to it having properties that would be valuable in a mnemonic system. These properties are *associativity*, *cooperativity*, and *input-specificity*. For a more extensive review of these features see Bliss and Collingridge, (1993).

1.7.1 The classical properties of LTP.

The property of associativity arises as ‘weak’ inputs can only be potentiated if they are active at the same time as a ‘strong’ input, such as tetanic stimulation, to a separate but convergent input (McNaughton et al., 1978; Levy and Steward, 1979). A related property is cooperativity. Experiments by McNaughton et al., (1978) found that increasing the duration of each current pulse during a tetanus resulted in more LTP than that achieved with less current, presumably because of activation of more fibres. Even though these two properties are sometimes considered separately, it does not mean that they are mutually exclusive.

‘Weak’ inputs are only potentiated if they are active at the same time as ‘strong’ inputs. However, the co-operative firing of a number of fibres may be sufficient to trigger the induction of LTP, despite the fact that any one of these inputs alone would be below the threshold for induction of LTP. LTP is also input specific as inputs which are active at the time of tetanic stimulation show potentiation, whereas inactive inputs do not (Andersen et al., 1977; Lynch et al., 1977). Input specificity need not mean literal synapse specificity (see 1.7.3).

These are seen as the classical properties of LTP which taken together suggest that LTP occurs at specific synapses at times of conjunctive pre- and post-synaptic activity, and this occurs during depolarisation of the postsynaptic cells to which they are connected.

The strong depolarisation required for LTP was thought to arise primarily from the temporal and spatial summation of EPSPs during the stimulation of numerous presynaptic fibres (Nicoll et al., 1988; Gustafsson and Wigstrom, 1990; Debanne et al., 1996). Recent findings indicate that action potentials initiated near the cell body back-propagate into pyramidal cell dendrites (Magee and Johnston, 1995; Spruston et al., 1995), suggesting that dendritic action potentials may provide an additional way of reaching the necessary level of postsynaptic depolarisation that is required for LTP induction. Markram et al., (1997) found that in pyramidal cells of the neocortex, increases in EPSP amplitude result when a postsynaptic neuron is depolarised to produce a burst of action potentials that coincides with a tetanic train of EPSPs. No increase was seen when pairing of high frequency EPSPs with a postsynaptic depolarisation sub-threshold for action potential initiation was performed, or when action potentials and EPSPs did not show some temporal conjunction (< 100 ms). Similar findings have been reported in the CA1 of the hippocampus (Magee and Johnston, 1997).

These recent experiments confirm the ‘classical’ properties of LTP. Nevertheless, over the years additional evidence has emerged that has shed new light on these ‘classical’ properties. Some of these ‘modern’ properties of LTP, have highlighted how synaptic plasticity may be modulated by prior activation of the same synapses during the induction of LTP, an idea central to the experiments reported here (for a more complete description of these ‘modern’ properties see Martin et al., 2000; Martin and Morris, 2002).

1.7.2 Post-synaptic bursting and ‘naturalistic’ patterns of stimulation.

Experiments described above (i.e. Kelso et al., 1986; Magee and Johnston, 1997; Markram et al., 1997) highlighted the role that back propagating action potentials play in induction of LTP. The classical properties of LTP, assert that afferent stimulation must be above a critical threshold to achieve sufficient depolarisation of the postsynaptic cell. However, as these new experiments highlight, presynaptic bursts that are conveniently used to induce LTP in most studies may not be the only way to induce LTP. In the hippocampus, bursts of postsynaptic action potentials can also contribute to the induction of potentiation *in vitro*, even if the presynaptic afferent stimulation is quite weak (Gustafsson et al., 1987; Thomas et al., 1998; Pike et al., 1999).

A further implication of these experiments concerns the way in which LTP is induced. Long trains of high frequency stimulation of presynaptic fibres used to induce LTP certainly do not mimic any kind of naturally occurring patterns of neural firing. Stimulation with more ‘naturalistic’ patterns i.e. theta-burst stimulation, has been found to induce LTP for several weeks *in vivo* (Larson et al., 1986; Rose and Dunwiddie, 1986; Staubli and Lynch, 1987). The theta rhythm can be induced in the CA1 of hippocampal slices by bathing the slice in the cholinergic agonist carbachol. Using this approach, Huerta and Lisman, (1993) reported that trains of single pulses each locked to the positive phase of the theta wave are sufficient to induce LTP. These experiments as a whole draw our attention to the way in which LTP is induced and the potential impact that this may have on subsequent processes that underlie the maintenance of potentiation.

1.7.3 Input-specificity may not imply synapse-specificity.

The degree to which the property of input specificity relates to the level of changes at individual synapses may also need revising. This comes from elegant experimental work by Engert and Bonhoeffer, (1997). Their findings question the degree to which synapse specificity is maintained during LTP induction. In these experiments, slice cultures were bathed in a low calcium solution that contained cadmium to prevent transmission throughout the entire slice. Focal applications of high calcium solutions were made during minimal presynaptic afferent firing to allow activity to occur only at a small number of local synapses. LTP was induced at the active synapses but also at nearby ($< 70 \mu\text{m}$) inactive synapses to the same cell.

1.7.4 'Metaplasticity'.

Several experiments by a number of groups have shown that prior tetanisation can inhibit the subsequent induction of LTP (Fujii et al., 1991; Huang et al., 1992b; Abraham and Hugget, 1997) and facilitate the induction of LTD (Wagner and Alger, 1995; Holland and Wagner, 1998). This has led to the idea that the magnitude and direction of the changes in synaptic efficacy, which are responsible for LTP, can be influenced by the prior history of synaptic activity. An interesting further point is that the prior synaptic activation may not itself induce any change in synaptic efficacy, but can still alter the capacity of a synapse to sustain plastic changes in the future. This interesting 'new' property has been termed 'metaplasticity' (Abraham, 1996; Abraham and Bear, 1996). This newly discovered property is even more interesting given the evidence that is now emerging concerning the role of phosphorylation and dephosphorylation of GluR1 AMPA receptor subunits following LTP and LTD (Lee et al., 2000).

1.7.5. Modulation of LTP.

In earlier sections, evidence was presented that suggests that the storage of information in memory can be modulated by various events at, or around the time of encoding. A similar modulatory effect on hippocampal LTP has been found. The mechanisms of heterosynaptic modulation of LTP are central to some of the questions addressed in the experiments reported in this thesis (see Chapters 5, 6, 7). Ikegaya et al., (1994) found that the magnitude of LTP in the medial perforant path-dentate granule cell synapse *in vivo* was attenuated by lesions to the ipsilateral, but not contralateral, basolateral amygdala (BLA). The BLA has a facilitatory effect on dentate gyrus LTP and this is dependent upon NMDA receptor activation within the BLA during a high frequency tetanus (Ikegaya et al., 1995b). Similar findings were subsequently reported in experiments where the BLA was temporally inactivated at the time of tetanic induction of LTP in the dentate gyrus (Ikegaya et al., 1995a). Inactivating the BLA after LTP had been induced (i.e., during the 'maintenance' phase) had no effect on established dentate gyrus LTP.

Experiments have also demonstrated that stimulation of various amygdaloid nuclei produce facilitatory effects on dentate gyrus LTP *in vivo*. Ikegaya et al., (1995c) found that single pulses of the medial amygdaloid nucleus (MeA) increased the amplitude of dentate gyrus population spikes when delivered simultaneously with perforant path pulses. When high frequency stimulation was applied to the MeA, a gradual increase in the perforant path

response was seen that was maintained for > 60 min. Subsequently, 'weak' tetanisation of the perforant path resulted in a very slight increase in the amplitude of the response. However, if the high frequency stimulation of the MeA occurred simultaneously, then robust LTP was induced. Similar results have been found with simultaneous BLA stimulation during a 'weak' tetanus to the perforant path (Ikegaya et al., 1996a). In order to try to identify the neurotransmitter involved in the effect that BLA stimulation has on LTP in the perforant pathway, Ikegaya et al., (1997) conducted experiments with β -adrenergic and cholinergic antagonists infused into the BLA. They found that injections of β -adrenergic but not cholinergic antagonists significantly attenuated perforant path LTP without affecting basal synaptic responses. Similar effects have been reported by other research groups (Akirav and Richter-Levin, 1999).

LTP is not observed when repetitive stimulation is at lower frequencies of ~ 10 Hz. In the behaving rat, a predominant component of hippocampal activity occurs near this 'neutral frequency' i.e., in the 5 – 12 Hz range, occurs during exploratory behaviour (O'Keefe and Nadel, 1978). Under certain situations extrinsic input to the hippocampus, in addition to intrinsic factors, may alter the frequency at which synapses are activated. Therefore, the rules governing the change in synaptic weights may be altered. Activation of β -adrenergic receptors in the hippocampus appears to be one such mechanism (Thomas et al., 1996; Katsuki et al., 1997). This is important as although under 'resting' conditions the activity of hippocampal neurons may be in this 5 – 12 Hz range, if something happens to the animal that is emotionally arousing, extrinsic inputs activated by such environmental events may alter the ability of such low level activity to induce changes in the active neurons.

Although β -adrenergic receptor activation does not seem to modulate the induction of LTP by high frequency stimulation (Sarvey et al., 1989), there is evidence that suggest that at lower frequencies, i.e., 5 – 10 Hz, these receptors can modulate LTP (Thomas et al., 1996). In these experiments (Thomas et al., 1996), hippocampal slices were stimulated at 5 Hz, for either very 'brief' (30 s) or 'prolonged' (3 min) periods. LTP was induced by 'brief' 5 Hz stimulation, but not by the 'prolonged' 3 min stimulus. However, if the β -adrenergic receptor agonist isoproterenol was applied before the 'prolonged' stimulus, LTP was now induced and there was no effect on the short 30 s, 5 Hz induced LTP in the presence of the drug. One possibility is that during long periods of 5 Hz stimulation, the processes responsible for the induction of LTP are initially activated, however, the sustained activity at this frequency may

trigger processes which oppose LTP, such as protein phosphatases. Activation of β -adrenergic receptors may overcome these inhibitory effects. The balance between protein phosphatase and kinase activity regulates the induction of either LTD or LTP (Coussens and Teyler, 1996). β -adrenergic receptor activation increases neuronal cAMP production in the CA1, and postsynaptic effects of β -adrenergic agonists have been attributed to increased cAMP in CA1 cells (Segal et al., 1981; Madison and Nicoll, 1986; Pedarzani and Storm, 1993). PKA phosphorylates the regulatory protein inhibitor-1, which in turn inhibits protein phosphatase 1 (PP1). Thomas et al., (1996) found that activation of the PKA pathway with forskolin, enabled the induction of LTP by the ‘prolonged’ 5 Hz stimulus, and this was inhibited by H-89, a PKA antagonist. Additionally, inhibition of PP1 with calyculin A mimicked the effects of isoproterenol on LTP induction with long periods of 5 Hz stimulation.

The mechanism by which this facilitatory effect of β -adrenergic receptor activation on low frequency induced LTP takes place, is beginning to unravel. Stimulation of large numbers of presynaptic fibres was originally suggested as the mechanism governing the depolarisation necessary for LTP induction (Nicoll et al., 1988; Gustafsson and Wigstrom, 1990; Debanne et al., 1996). Recent experiments highlight how action potentials initiated near the cell body back-propagate into pyramidal cell dendrites (Magee and Johnston, 1995; Spruston et al., 1995), and this may be another way of achieving sufficient levels of depolarisation. Bursts of action potentials enable the induction of LTP by theta burst stimulation in hippocampal slices *in vitro* (Thomas et al., 1998). Field recordings showed that theta burst stimulation alone (150 stimuli at 10 Hz, which resulted in no LTP) was not associated with either single or complex spikes. However, stimulation in the presence of isoproterenol resulted in the generation of complex spikes (Brown et al., 2000). Therefore, although sufficient depolarisation may not result from theta burst stimulation alone, with β -adrenergic receptor activation such conditions may be met by triggering action potentials that back-propagate into the dendrites. This effect of β -adrenergic receptor activation was associated with phosphorylation of inhibitor-1, and reduction in the activity of PP1. Substituting isoproterenol with phosphorylated inhibitor-1, was found to mimic the effect on theta burst stimulation on inducing LTP (Brown et al., 2000). In later sections, emphasis is placed on the role of CaMKII in the induction of LTP. CaMKII is a substrate for PP1 (Strack et al., 1997), when the same experiments with pairing of β -adrenergic receptor activation with theta burst stimulation occurred while intracellular application of a selective CaMKII inhibitor was

conducted, no facilitatory effect was now seen. This was also the case when a PP1 inhibitor was present, leading the authors to suggest that PP1 activation is upstream of CaMKII.

1.7.6 Behavioural modulation of LTP.

However, electrical stimulation is not the only means of augmenting LTP in the dentate gyrus. In a series of studies, Seidenbecher et al., (1995, 1997) found that LTP induced by a 'weak' tetanus (which normally results in decremental perforant path LTP) could be modulated by the 'behavioural state' of the animal at the time of tetanisation. Water-deprived rats that were allowed to drink during or after a 'weak' tetanus showed more robust LTP compared to non-deprived controls. This effect was blocked by infusions of β -adrenergic receptor antagonists infused intraventricularly before LTP induction. In a recent study, Frey et al., (2001) found that the induction of a normally short-lasting LTP with 'weak' tetanic stimulation to the perforant path was augmented by a reinforcing high frequency tetanus to the BLA. This effect was found to occur if the BLA tetanus either preceded, or followed, the perforant path tetanus by 5 or 15 min. When AP5 was applied intraventricularly 10 min after the perforant path tetanus, and 5 min before the BLA tetanus, the LTP induced was not affected. However, similar timing of the injection schedules for a number of drugs (a β -adrenergic, muscarinic, and dopaminergic receptor antagonist) showed that only the β -adrenergic antagonist blocked the effect that BLA stimulation had on LTP in the dentate gyrus. No 'rescue' of persistent LTP induced with a 'weak' tetanus occurred if a protein synthesis inhibitor had been injected.

1.8 NMDA receptor dependent LTP.

The 'classical' properties of LTP that have been discussed highlight some of the factors that govern the induction of LTP. Most of the evidence discussed relates to LTP in the hippocampus that is dependent on NMDA receptor activation. However, not all forms of LTP are glutamatergic, or NMDA receptor dependent, and the hippocampus is not the only brain region in which LTP can be induced. Since the experiments discussed here concern CA1 hippocampal LTP, attention shall be largely focussed on this form of LTP (for more details concerning the pre- and postsynaptic mechanisms, signal transduction pathways, and molecular mechanisms see Johnston et al., 1992; Bliss and Collingridge, 1993; Nicoll and Malenka, 1995; Fregnac, 1997). LTP is not the only form of activity dependent synaptic change that has been described, the opposite i.e., a weakening of synaptic strength, or long-

term depression (LTD) has also been the focus of rigorous study. Unfortunately, as the phenomenon of LTD is not directly relevant to the experiments reported here, very little mention of LTD is made, (see Linden and Connor, 1995; Bear and Abraham, 1996 for reviews).

1.8.1 Calcium and the NMDA receptor.

Lynch et al., (1983) found that injection of the calcium chelator EGTA into the postsynaptic cell prevented the induction of LTP. This finding focussed attention on the role that increases in postsynaptic calcium (Ca^{2+}) may play in the initial stages of induction of LTP. Limiting the magnitude of the rise in postsynaptic Ca^{2+} during the induction of LTP only results in a very transient short-term potentiation (i.e., potentiation that persists for 5-20 min), (Malenka, 1991; Hanse and Gustafsson, 1992; Malenka et al., 1992). It was thought that this rise in intracellular Ca^{2+} might be sufficient to generate LTP via a postsynaptic modification. However, Gustafsson et al., (1987) found that depolarising the postsynaptic cell for 2 min at –40 mV, thus causing continuous Ca^{2+} spiking due to Ca^{2+} entry via dendritic voltage-dependent Ca^{2+} channels failed to cause any increase in synaptic transmission. Also repetitive and prolonged activation of voltage-dependent Ca^{2+} channels in the absence of synaptic stimulation can potentiate the responses, but only transiently as within 30-40 min of such stimulation the response always decays to basal levels (Kullmann et al., 1992).

Even though the levels of intracellular Ca^{2+} generated by such manipulations would be considerable (the response was potentiated 3-4-fold in the initial stages), it appears that rises in intracellular Ca^{2+} alone are not sufficient to generate more lasting potentiation. It is clear, that in order to see any observable changes in the efficacy of synaptic transmission, activation of the NMDA receptor is critical. Given that the NMDA receptor is permeable to Ca^{2+} , (MacDermott et al., 1986; Ascher and Nowak, 1988), and that the NMDA receptor antagonist AP5 blocks LTP at CA1 synapses (Collingridge et al., 1983), this particular receptor appears to be of particular importance in the induction of LTP.

1.8.2 Properties of the NMDA receptor.

The *N*-methyl-D-aspartate (NMDA), receptor has a number of characteristics that are of importance for the role it plays in the induction of LTP. The NMDA receptor (NMDAR) channel is gated in a unique manner both by ligands and by voltage. The voltage dependence

is caused by Mg^{2+} block within the ion channel (Mayer et al., 1984; Nowak et al., 1984). It is also different from other non-NMDA channels in regard to the excitatory postsynaptic potential mediated by this channel, as the EPSP displays a prolonged time course relative to non-NMDA channels (Dale and Roberts, 1985).

During basal synaptic transmission (at the Schaffer-collateral/CA1 synapse) glutamate release from presynaptic terminals binds to both NMDA and α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors located on the dendritic spines of the postsynaptic cell. Due to the presence of the Mg^{2+} block within the ion channel, sufficient depolarisation is needed to remove this block. At resting membrane potential (-70 mV) the current generating the synaptic response flows via the AMPA receptor channel (Collingridge et al., 1983), which can be blocked by the addition of CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione), (Andreasen et al., 1989).

1.8.3 Activation of the NMDA receptor.

It was mentioned above that the NMDAR shows a slow decay time course for the EPSPs which it is involved in generating. This is primarily due to the slow activation and deactivation kinetics of these receptors (Lester et al., 1990). This fact is relevant to the discovery that during low frequency stimulation a small proportion of NMDA receptors do see a relief of the Mg^{2+} block. However, by the time a significant number of channels are open to depolarise the cell sufficiently, the membrane is already in a hyperpolarised state (due to concurrent inhibition from GABA-ergic interneurons), and this increases the Mg^{2+} block on the receptors even further (Collingridge et al., 1988).

Evidence using calcium imaging to monitor calcium transients in individual dendritic spines has shown that NMDARs are activated in response to single stimuli in cerebellar purkinje cells (Denk et al., 1995; Eilers et al., 1995), and in hippocampal pyramidal cells (Malinow et al., 1994; Yuste and Denk, 1995). Activation of ionotropic glutamate receptors is necessary for the observed synaptically evoked calcium transients in dendritic spines (Muller and Connor, 1991; Alford et al., 1993). Emptage et al., (1999), demonstrated that during basal synaptic transmission the NMDA receptor evokes substantial calcium release from internal stores without inducing LTP. On the other hand, during tetanic stimulation there is a greater and long-lasting depolarisation, which alleviates the Mg^{2+} block of NMDA channels.

1.8.4 Pre- or post-synaptic changes and LTP.

The properties of the NMDA receptor that have been discussed above provide a scheme in which many researchers have set about investigating the mechanisms involved in the expression of LTP. Depolarisation of NMDA receptors, and the concurrent changes in the level of Ca^{2+} , appear to be important factors in the induction of LTP. LTP is then thought to be expressed, at least in part, by changes in α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor function (Kauer et al., 1988b; Muller and Lynch, 1988; Davies et al., 1989). However, an important question that has dominated much research concerns the locus of expression of LTP. Is the change in synaptic strength that occurs during LTP due to a pre- or post-synaptic modification?

Evidence in support of either a pre- or post-synaptic mechanism is available, although LTP expression and maintenance may involve both. Many studies point to post-synaptic changes, such as a change in response to transmitter release as indicated by changes in the amplitude of miniature EPSCs (mEPSCs), (Manabe et al., 1992). Although, a consistent finding in many experiments is that there is a reduction in the number of synaptic failures after LTP (Malinow and Tsien, 1990; Kullmann and Nicoll, 1992; Stevens and Wang, 1994; Isaac et al., 1995; Liao et al., 1995), initially presumed to arise from a failure in transmitter release (i.e., a presynaptic change).

1.8.5. Pre-synaptic expression mechanisms.

Potential presynaptic changes which may account for the associated increase in synaptic strength associated with LTP include enhanced transmitter release (due to changes in quantal content), the number of vesicles released, or changes in the probability of release. Early evidence for presynaptic mechanisms involved measuring transmitter release following LTP. Pre-accumulated [^3H]-D-aspartate release from presynaptic terminals was significantly increased in response to bursts of electrical stimulation (Skrede and Malthe-Sorensen, 1981). Early *in vivo* evidence from studies involving the induction of LTP showed that using a push-pull cannulae technique to measure [^3H]-glutamate release, glutamate levels increased and remained elevated for at least 1 hr (Dolphin et al., 1982), although others report no such changes (Aniksztejn et al., 1989).

If there is a change in the amount of glutamate released both AMPA and NMDA receptors ought to be equally affected (both are located in close proximity in the postsynaptic

membrane, (Bekkers and Stevens, 1990), with a parallel increase in the size of the both AMPA and NMDA receptor mediated response. Various presynaptic manipulations such as increasing the number of active synapses by increasing the stimulus strength did cause a parallel increase in both receptor-mediated components. However, after LTP only the AMPA receptor mediated component was altered (Kauer et al., 1988b). Other studies looking at changes in paired-pulse facilitation (Selig et al., 1999), saturating the amount of transmitter released (Hjelmstad et al., 1997), and monitoring transmitter release via glial glutamate transporter currents (Luscher et al., 1998), all suggest that LTP is not associated with an increase in transmitter release, either as a consequence of an increase in the probability of release or in the number of synapses releasing vesicles.

1.8.6 Post-synaptic expression mechanisms: Silent synapses.

An increase in the probability of release is associated with a reduction in the number of synaptic failures, and experiments using minimal stimulation have shown that LTP is associated with an increase in this probability (Kullmann and Nicoll, 1992; Isaac et al., 1996). The discovery of postsynaptic ‘silent’ synapses and the demonstration that these could be converted into active synapses by postsynaptic modifications suggests a resolution to many of the arguments concerning a pre- versus post-synaptic explanation of the expression of LTP (Isaac et al., 1995; Liao et al., 1995). During basal synaptic transmission, synapses are said to be ‘silent’ if they show an NMDA but no AMPA mediated response. In the resting state, NMDA receptors are minimally opened and transmitter release at such a synapse results in a synaptic failure. During LTP, AMPA receptors are recruited at the active synapses, with no change in the NMDA response but reducing the rate of failures. This is suggestive of a postsynaptic modification involving the recruitment of functional AMPA receptors.

Indirect evidence, that is suggestive of such a mechanism, came from studies with hippocampal slices. Kullmann, (1994) showed that the coefficient of variation (CV, i.e. the mean divided by the standard deviation) was larger in the AMPA mediated receptor current than in the NDMA current. LTP induction caused a decrease in the CV of the AMPA current, but had no effect on either the CV or the average amplitude of the NMDA current. The CV is inversely proportional to the number of activated synapses, leading the author to conclude that the results could be explained if AMPA receptors were absent, or non-functional in a proportion of synapses, and that after LTP insertion or activation of AMPA receptors occurred in the postsynaptic membrane.

Much evidence in favour of such a mechanism involving insertion of AMPA receptors into the postsynaptic membrane has come from molecular and electrophysiological experiments with recombinant receptors. AMPA receptors come in different forms, but all interact with different cytoplasmic proteins through their C-terminal tails. Many of the proteins that they have been found to interact with are rich in PDZ domains, such as SAP97 (Leonard et al., 1998), and glutamate receptor-interacting protein GRIP, (Dong et al., 1997). The AMPA receptor subtype GluR2, has also been shown to interact with NSF (NEM-sensitive factor), (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998) which is known to play an important role in membrane fusion processes that underlie intracellular protein trafficking and presynaptic vesicle exocytosis (Rothman, 1994). These interactions with these types of proteins suggest that AMPA receptors contain the molecular machinery to enable their trafficking to the postsynaptic membrane.

The electrophysiological evidence that has looked at the contribution of AMPA receptor location to synaptic transmission is based on experiments with electrophysiologically tagged recombinant AMPA receptors. These experiments are based on the property of rectification, i.e., the basic property of a receptor that is detected as the ratio of the response observed at – 60 mV to that at + 40 mV. Endogenous AMPA receptors, which mainly contain the GluR2 subunit, pass current equally well in both directions. Utilising this approach Hayashi et al., (2000), have demonstrated that LTP and over-expression of CaMKII induce the delivery of GluR1-containing receptors into synapses. An interaction between GluR1 and a PDZ-domain protein is necessary for LTP or CaMKII to drive synaptic delivery of GluR1, as point mutations in the PDZ-binding region of GluR1 prevent its synaptic delivery. Other evidence has looked at delivery of endogenous receptors. Heynen et al., (2000), found that following LTP in the hippocampus *in vivo*, the amount of GluR1 and GluR2 was increased in synaptoneurosomes, and this was blocked by AP5. In cultured neurons, LTP of miniature excitatory postsynaptic currents could be triggered following brief pharmacological stimulation of NMDA receptors and this was accompanied by a rapid insertion of endogenous AMPA receptors (Lu et al., 2001).

1.8.7 The 'spillover hypothesis'.

The evidence in favour of a postsynaptic locus of expression of LTP appears to be growing substantially. However, one other possible explanation that suggests a pre-synaptic

mechanism comes from the ‘glutamate spillover hypothesis’ (Kullmann et al., 1996; Kullmann and Asztely, 1998). In experiments with hippocampal slices, NMDA receptors have been found to consistently sense a larger number of quanta of glutamate released from presynaptic terminals than do AMPA receptors. One possibility is that as described above, namely, a proportion of synapses express functionally silent synapses (although they can be uncovered by LTP). Alternatively, glutamate may spill over from neighbouring synapses and selectively activate NMDA (but not AMPA) receptors.

Whether or not LTP is expressed exclusively presynaptically or postsynaptically (or a combination of both) is still open to debate. A more significant question that will be addressed in the following sections concerns the critical downstream targets of NMDA receptor activation following the induction of LTP. Although presynaptic changes may also be involved in these events, most of the evidence reviewed will focus on the postsynaptic signalling pathways that have been implicated in NMDA receptor dependent LTP.

1.9 The distinction between early-LTP (E-LTP) and late-LTP (L-LTP).

Although a direct mapping of a physiologically unnatural process such as LTP (at least in terms of how LTP is usually induced) onto the phenomenon of memory is too simplistic, one must consider that as memory has been shown to display different stages (see Section 3. Short-term and long-term memory), one might expect a similar requirement of LTP. A variety of different experimental approaches have demonstrated that it is possible to mechanistically distinguish between at least two (possibly three) temporal ‘phases’ of LTP, i.e., early-LTP (E-LTP) and late-LTP (L-LTP).

1.9.1 Induction, expression and maintenance of LTP.

Over the years a picture has emerged that suggests that there are a number of different ‘stages’ of LTP. It is important to distinguish between the induction and expression of E-LTP, as induction mechanisms have been defined as those processes whose inhibition blocks LTP when an inhibitor is applied only during the inducing stimulus and then washed out. Expression mechanisms are brought about by the induction mechanisms, but are distinguished on the basis that interfering with expression mechanisms after the inducing stimulus causes

established E-LTP to be diminished (Roberson et al., 1996). The activation of ‘expression mechanisms’ may be crucial for the longer maintenance phases of LTP (i.e., L-LTP).

These distinctions are relevant to the discussion of second messenger systems that have been suggested to play different roles in the two main subtypes of LTP. The presence of an inhibitor throughout the duration of an experiment (or the absence of the molecule, for example, in a knockout experiment) will complicate the distinction being made in terms of induction or expression mechanisms. Additionally, although activation of expression mechanisms is crucial for later stages of LTP (i.e., maintenance) it may not be the case that these mechanisms must persist for a definite period in order to give rise to maintained LTP. Expression and maintenance mechanisms may be triggered in parallel.

1.9.2. Signalling downstream of NMDA receptor activation.

Following the activation of NMDA receptors and the resulting calcium influx a number of downstream signalling cascades may be engaged. Immediately following tetanic stimulation to induce LTP, if the broad spectrum kinase inhibitor H-7 is applied during the tetanus, only a decremental potentiation lasting about 30 min is observed (Malinow et al., 1988). This initial phase of LTP (usually referred to as STP, or short-term potentiation) has been shown to be unaffected by a number of different protein kinase inhibitors (Lovinger et al., 1987; Reymann et al., 1988a; Malinow et al., 1989; Colley et al., 1990; Denny et al., 1990; Muller et al., 1990; Reymann et al., 1990; Matthies et al., 1991; Wang and Feng, 1992).

Many different molecules have been reported to have some part in the conversion of the initial calcium signal into a long-lasting change that underlies the expression of LTP. In a recent review Sanes and Lichtman, (1999) list over 100 molecules which have been found to affect LTP in one way or another. Molecules implicated in the initial induction and expression of LTP include glutamate receptors, intracellular messengers, ion channels, vesicle and synapse-associated proteins, adhesion molecules, proteases, phosphatases, phospholipases, and kinases. These molecules may play some part in either induction or expression mechanisms, however there are additional complications. Any of these same molecules may not be necessary for the occurrence of LTP but may rather modulate either its induction or expression. Alternatively, they may have intermediate effects on processes that may affect LTP indirectly (such as metabolic or growth processes) without ‘directly’ mediating the processes involved in either induction or expression. As so many different molecules have been implicated in LTP in some way, discussion is limited to those that appear to be most

relevant to the core ideas of the experiments reported in this thesis. Therefore, discussion is limited to the role of PKC, CaMKII, PKA, and MAPK in the two main phases of LTP, early- and late- LTP.

Although separate sections are devoted to discussing the possible role of each of these protein kinases in LTP, this is not meant to imply that there are completely independent of each other. On the contrary, there may be considerable cross talk between many of the proteins that have been identified to play some role in the induction and maintenance of LTP. Evidence shall be presented which suggests how this may occur.

1.9.3 Autonomous activity of protein kinases.

Immediately following STP, a longer phase of LTP called early-LTP (E-LTP) is initiated and is usually expressed for approximately 2-3 hr after the inducing stimulus. A number of calcium dependent kinases have been shown to influence synaptic transmission and LTP, including protein kinase C (PKC), cAMP-dependent protein kinase A (PKA), Ca^{2+} /calmodulin-dependent protein kinases (CaMKII and IV), cGMP-dependent protein kinase (PKG), and also a number of receptor tyrosine kinases such as *fyn* (see Schulman, 1991; Walaas and Greengard, 1991 for reviews).

Lisman, (1985) described how a kinase that, upon autophosphorylation is autonomously activated, can become permanently active even after termination of the initial triggering event. Both protein kinase C (PKC), (Huang and Huang, 1986) and calcium/calmodulin-dependent kinase II (CaMKII), (Miller and Kennedy, 1986) display this property.

1.9.4 Protein kinase C (PKC) and E-LTP.

It is crucial to distinguish between effects on the induction or the expression of E-LTP. Malinow et al., (1989) found that in experiments in which simultaneous extracellular and intracellular recordings were made while either H7 or PKC inhibitory peptides (PKC_{19-31}) were injected into individual postsynaptic cells, either manipulation blocked the induction but not the expression of E-LTP. This question of the role of PKC in either only the induction or expression, or possibly both stages of LTP, is quite controversial. There are a number of different PKC isoforms, (for a review see Tanaka and Nishizuka, 1994). They are composed of two regions, a regulatory subunit and a catalytic subunit. In the absence of second

messenger activators (such as, calcium, diacylglycerol, phorbol esters), the kinase is inactive because the regulatory portion of the enzyme masks the catalytic site.

The conclusion from a number of studies is that there is a critical time window, activated at the time of tetanus until just shortly after, in which PKC inhibitors block only the induction and not the expression of LTP (see Lovinger et al., 1987; Malinow et al., 1988; Reymann et al., 1988; Colley et al., 1990; Muller et al., 1990; Reymann et al., 1990). To take the first experiment as an example, in the dentate gyrus *in vivo*, iontophoretic injections of inhibitors that act either on the regulatory domain of PKC (such as Polymyxin B), or inhibitors that act on the catalytic domain (such as H-7), blocked only the expression but not the induction of LTP (Lovinger et al., 1987). However, some experiments have shown that delayed application of various PKC inhibitors or PKC inhibitory peptides, can reverse pre-established LTP. The non-specific PKC inhibitor staurosporine has been shown to reverse pre-established LTP when applied 90 min after the tetanus (Matthies et al., 1991), and a intracellular injection of a cocktail of both the PKC inhibitory peptide (PKC₁₉₋₃₁) and polymyxin B three hours after tetanisation caused LTP to return to baseline (Wang and Feng, 1992).

Klann et al., (1991) demonstrated that expression of LTP is associated with an increase in PKC activity in hippocampal slices. Subsequently, using a peptide substrate selective for PKC, the same group has shown that PKC activity was increased 45 min after LTP, and this increase was blocked by the inhibitory peptide (PKC₁₉₋₃₁) and AP5 (Klann et al., 1993). It is intriguing that they found that this increase in PKC activity was evident immediately after the tetanus, and remained elevated for up to 2 – 3 hr. Other non-selective PKC substrate studies have also shown increases in PKC activity following LTP (Akers and Routtenberg, 1985; Lovinger et al., 1985; Nelson et al., 1989).

1.9.5 Autonomous activation of PKC.

In the above study of Klann et al., (1993) it was shown that increased PKC activity was observed to remain elevated for 2 – 3 hr after LTP induction. As the initial calcium signal that activates PKC is transient, a mechanism for generating autonomous activity of PKC must be possible. Lisman, (1985), described how a kinase that upon autophosphorylation is autonomously activated, and subsequently permanently active even after termination of the initial triggering event.

Autonomous activation may arise via three different mechanisms; (1) As a result of proteolysis, whereby the regulatory domain of the PKC subunit is removed leaving only the catalytic domain (PKM), and this remains active in the absence of second messengers (Huang and Huang, 1986). An increase in the proteolytic fragment of PKC has been observed after the induction of LTP *in vitro* (Sacktor et al., 1993). (2) Another mechanism for generating autonomous activation is insertion of active PKC from the cytosol into the cell membrane. Akers et al., (1986), observed an increased membrane association of PKC in LTP. (3) The final mechanism for generating autonomous activity is autophosphorylation. Autophosphorylation of PKC in the presence of calcium and phospholipid is known to occur (Huang and Huang, 1986; Flint et al., 1990). The evidence of Klann et al., (1993), discussed above, is suggestive of this mechanism being involved in LTP. They ruled out the proteolytic mechanism as no low molecular weight fragments corresponding to the constitutively activate catalytic fragment (i.e., PKM), were found in Western blots.

1.9.6 Atypical PKC's.

It is important to distinguish between an inhibitory effect on processes responsible for the very early stages of induction of LTP and those processes responsible for expression of E-LTP. In all of the studies listed above, either with cell-permeant inhibitors or inhibitory peptides, inhibition of PKC begun either before, or at the time of tetanisation. Therefore, it is difficult to rule out a role of PKC in the initial stages of E-LTP expression. Although some initial potentiation was induced in all of these studies, LTP was not found to last for the 2-3 hr period, which has been suggested to be a characteristic feature of E-LTP.

Additionally, the complication in terms of the relative importance of PKC in either the induction or expression of LTP, or possibly both, is not helped by the fact that the different inhibitors used in these studies act in different ways. The evidence from Klann et al., (1993), and also the findings of Sacktor et al., (1993), may help clear up some of this controversy.

Both studies show that PKC is active during the expression/early-maintenance phase of LTP (i.e., 30 – 180 min after LTP induction). Both studies found evidence for a persistently active form of PKC with atypical properties, that is, the enzyme is independent of calcium, and diacylglycerol. Sacktor et al., (1993) provide evidence to showing that this atypical enzyme is PKM ζ . The three mechanisms for generating autonomous PKC were briefly described above; proteolytic cleavage would result in formation of PKM ζ . However, Klann et al., (1993)

suggest that autophosphorylation is the required mechanism. It is possible that the antibody used to detect changes in PKC activity by Klann et al., (1993) was not able to detect PKM ζ (Schwartz, 1993). If formation of PKM ζ is the mechanism for generating autonomously active PKC, then failure to see an inhibitory effect on established LTP, can be explained as the target of these inhibitors (i.e., the regulatory domains) in the intact PKC molecule are absent in PKM ζ and so can no longer affect PKC activity.

Very recently, evidence implicating the catalytic domain of PKM ζ in both the maintenance of memory and LTP has been reported (Drier et al., 2002; Ling et al., 2002). Long-term memory for odour-avoidance was studied in *Drosophila* in which the persistently active PKM ζ transgene was expressed under the control of a heat-shock promoter (Drier et al., 2002). They found that induction of this transgene after training enhanced memory tested 24 hr later. Further, a specific PKM ζ inhibitor chelerythrine, or induction of a dominant negative transgene caused faster forgetting, without any effects on the ability to learn.

In the other study, this time using CA1 hippocampal slices, the authors found that exogenous application of recombinant PKM ζ into the postsynaptic cell produced LTP that occluded the subsequent induction of tetanus induced LTP (Ling et al., 2002). The potentiation was also reversed by the same selective PKM ζ inhibitor, chelerythrine, used in the *Drosophila* experiment. They also found that the general kinase inhibitor staurosporine blocked the expression, and early maintenance of LTP when applied before tetanisation, but a delayed application 60 min after the tetanus had no effect. This was in contrast to chelerythrine which when applied 60 min after the tetanus completely reversed LTP that was expressed at 60 min, but was not maintained for the rest of the experiment, i.e., 5 hr. No effects were seen on a control pathway at any time. These results are suggestive of a mechanism whereby persistent kinase activity maintains LTP for a number of hours after the initial induction. However, PKM ζ may also be involved in the initial induction of LTP as the introduction of chelerythrine or a dominant negative inhibitor of PKM ζ into the postsynaptic cell completely blocked LTP induction.

Therefore, the controversy concerning the relative importance of PKC in the induction and/or the expression and early maintenance of LTP is not yet resolved. It seems more likely that different proteins and second messengers act in concert depending on the induction parameters used to trigger LTP. This may be due to the different

phosphorylation/dephosphorylation states of various mediators that may influence the persistence of various signals that are activated by the induction of LTP (see Section 1.10.9. The role of phosphorylation and dephosphorylation of AMPA receptors by CaMKII).

1.9.7. Mitogen-activated protein kinase (MAPK) and LTP.

Another group of proteins implicated in LTP are the mitogen-activated protein kinases. These enzymes regulate a wide array of cellular responses, such as cell proliferation and determination. English and Sweatt, (1996) found that p42 MAPK (extracellular signal-regulated kinase 2) is activated during the induction of LTP in CA1 hippocampal slices. Subsequently, the same group showed that using PD098059, an inhibitor of MEK (the protein kinase responsible for phosphorylating and activating MAPKs), the induction of LTP was blocked without any effects on basal synaptic transmission or NMDA receptor-mediated transmission (English and Sweatt, 1997). If the drug was applied 30 min after LTP induction, no effect on established LTP was observed.

However, others have questioned the relative importance of the MAPK pathway in comparison to other signalling pathways in the induction of LTP. Liu et al., (1999) suggest that at the concentration of PD098059 (50 μ M) used in previous studies by English and Sweatt, (1997) inhibitory effects on CaMKII phosphorylation were also observed. Lowering the concentration to 30 μ M had no effect on LTP but completely blocked p42 MAPK phosphorylation. Problems in interpreting these results again arise as the inhibitors are applied to the slices for long periods (up to 90 min pre-tetanus in the experiments of Liu et al., 1999) therefore the block after ~ 60 min may result from initial low levels of expression at the time of the tetanus.

Experiments by Giovannini et al., (2001) may suggest some resolution to these discrepancies. They found that with an LTP induction protocol where the application of the β -adrenergic agonist isoproterenol was paired with a theta-pulse stimulation protocol (TPS-ISO) LTP was induced. This theta-pulse stimulation does not, on its own cause any change in synaptic strength, but in the presence of β -adrenergic agonists, these drugs ‘modulate’ the ability of such stimulation to induce changes in synaptic strength. Application of 50 μ M PD098059 blocked both p42 MAPK phosphorylation and CaMKII phosphorylation. However, TPS-ISO induced LTP did result in a significant increase in total CaMKII levels in the CA1 that was blocked by PD098059, which according to the authors may suggest that transient activation of

the MAPK pathway contributes to a later increase in CaMKII perhaps by regulating translation of CaMKII. Anisomycin blocked the TPS-ISO induced rise in total amount of CaMKII in CA1. This is one possible way in which activation of one signalling cascade may also trigger the recruitment of other pathways, which enable the mechanisms underlying the persistence of LTP to outlast the initial triggering event.

1.9.8 Calcium/calmodulin dependent protein kinase II (CaMKII) and E-LTP.

Another protein that has been shown to undergo persistent activation in the absence of the initial triggering stimulus is calcium/calmodulin-dependent protein kinase II (CaMKII). There is an overwhelming amount of evidence showing that CaMKII is involved at some point in both LTP and memory, the most relevant evidence is discussed in the following sections (for more detailed discussions see Lisman, 1994; Malenka and Nicoll, 1999; Soderling et al., 2001; Lisman et al., 2002).

CaMKII is highly expressed in the hippocampus where it comprises ~ 2% of the total protein content (Erondy and Kennedy, 1985). There are a number of different CaMKII isoforms derived from four genes (α , β , γ , and δ). Each isoform consists of a catalytic domain, an autoinhibitory domain, and two other regions. Upon activation by calcium and calmodulin, and exposure of the single amino acid Thr286 on the autoinhibitory domain CaMKII can be autophosphorylated and rendered independent of calcium and calmodulin. Fukunaga et al., (1993) originally found that calcium-independent CaMKII activity is persistently elevated for at least one hour after LTP induction. Later, this was found to be accompanied by phosphorylation of CaMKII on Thr286/287 (Barria et al., 1997b).

Membrane permeant inhibitors of CaMKII, (KN-62) block the Ca^{2+} -dependent activity of the enzyme, and block LTP induction (Ito et al., 1991). In experiments that used a pairing protocol to induce LTP, rather than tetanic stimulation, in order to rule out a possible effect of CaMKII antagonists on processes responsible for postsynaptic depolarisation, LTP induced in this way was completely blocked by CaMKII inhibitors (Otmakhov et al., 1997). Experiments with transgenic animals in which genes encoding CaMKII have been deleted have already been discussed in Section 1.6.3., but these also help strengthen the link between CaMKII and induction of LTP.

1.9.9 CaMKII and activation of AMPA receptor-mediated transmission.

Again, the evidence concerning whether CaMKII is involved in the expression or only the induction of LTP is somewhat controversial (Feng, 1995). Given that it was known that CaMKII upon autophosphorylation of Thr286, could promote further autophosphorylation of the kinase, a positive feedback process may maintain its activity (Lisman, 1985; Lisman, 1994; Lisman and Zhabotinsky, 2001). If CaMKII remains persistently active, and this can be triggered by LTP (Barria et al., 1997b), one may suspect that LTP would be reversed if CaMKII activity is inhibited after its induction.

However, early work infusing CaMKII inhibitors in the postsynaptic cell through microelectrodes has given contradictory results (Malinow et al., 1989; Malgaroli et al., 1992; Feng, 1995). The involvement of CaMKII in both the induction and expression of LTP may only be expected if CaMKII acts alone. If an early activation of CaMKII occurs via autophosphorylation, and this leads to different effects on the same pathways activated by other proteins, then the job of triggering the expression and early maintenance of LTP may be passed over to other pathways. CaMKII may regulate the phosphorylation of AMPA receptors, thus altering their channel properties. This results from the effects that CaMKII has on the relative phosphorylation and dephosphorylation of specific sites on AMPA receptor subunits. If CaMKII activity is inhibited after LTP induction, no effect on the expression, or even maintenance of LTP may be seen if it acts in concert with other similar proteins (such as PKA and PKC), and there is some evidence that these molecules do share some points of overlap. cAMP is known to enhance the responses mediated by AMPA receptors in cultured hippocampal cells (Greengard et al., 1991).

The evidence for a postsynaptic expression mechanism via unmasking of ‘silent synapses’ and the resultant insertion of functional AMPA receptors into the synapse was presented earlier. In experiments with organotypic hippocampal slice cultures, viral infection with the AMPA subunit, GluR1 (tagged with green fluorescent protein, GFP) has shown that GluR1 is delivered to active spines following induction of LTP and that this delivery was blocked by addition of AP5 to the bath (Shi et al., 1999). Earlier sections discussed how changes in parameters such as quantal size and failure rate might be explained by the conversion of previously silent synapses into active synapses. A truncated, constitutively activated form of CaMKII was perfused directly into CA1 cells, and this resulted in a gradual increase in the size of the excitatory postsynaptic currents. The mechanism by which this occurred was found

to have similar effects on both an increase in quantal size and a reduction in failure rate, as that observed after LTP induction with tetanic stimulation (Lledo et al., 1995). Occlusion experiments verified that the effects of CaMKII were related to LTP. Whole-cell recordings of cells from the population that displayed stable LTP, showed that CaMKII applied to the whole-cell pipette solution increased the size of the response on the control pathway, but did not change the pathway expressing LTP.

Recent advances with the use of CaMKII labelled with GFP has helped researchers understand how CaMKII can move into synapses where it may exert its effects on LTP by means of interacting with various AMPA receptor subunits such as GluR1. In cultured hippocampal neurons, in which CaMKII isoforms were tagged with GFP, Shen and Meyer, (1999) found that either glutamate stimulation, or brief tetanic stimulation, resulted in translocation of GFP-CaMKII to the postsynaptic density, and that this is a calcium dependent process. This translocation was found to occur independently of autophosphorylation of CaMKII, as cultured neurons from the autophosphorylation deficient mutant T286A showed similar movement of tagged CaMKII into the post-synaptic density (Fong et al., 1989; Hanson et al., 1989).

1.9.10 Phosphorylation and dephosphorylation of AMPA receptors by CaMKII.

If CaMKII is capable of translocating to the post-synaptic density where the receptors involved in expressing LTP are located, once there, what occurs? The first clues come from experiments that have demonstrated that AMPA receptors are phosphorylated by CaMKII after LTP induction in hippocampal slices (Barria et al., 1997b). In both HEK-293 cells (Barria et al., 1997a), and in hippocampal slices (Mammen et al., 1997), it has been shown that the site on the GluR1 subunit that is phosphorylated by CaMKII is the Ser-831 residue. This was an interesting discovery as it has been known that PKC phosphorylates this subunit on Ser-831 also (Roche et al., 1996). Given the evidence discussed previously concerning the possible roles that PKC might play in LTP induction, the fact that both of these proteins phosphorylate the same AMPA receptor subunit on the same region is intriguing and suggest one way in which these proteins may interact to control LTP induction. Again in HEK-293 cells, it has been reported that out of a number of potential mechanisms for potentiating peak current amplitude upon phosphorylation of GluR1 by CaMKII, only an increase in channel conductance was found (Derkach et al., 1999).

More recently, elegant experiments by Lee et al., (2000), have looked at the relative role of phosphorylation and dephosphorylation of the GluR1 subunit following both LTP and LTD. In hippocampal slices, using specific antibodies against GluR1, LTD was found to be associated with specific dephosphorylation of Ser-845, whereas there was no change in phosphorylation of Ser-831. Ser-845 is the GluR1 site that is specifically phosphorylated by PKA. Incubating the slices in a protein phosphatase inhibitor (okadaic acid) both abolished LTD, and the associated dephosphorylation of Ser-845. On the other hand, LTP was associated with a specific increase in GluR1 phosphorylation at Ser-831, with no change this time in phosphorylation of Ser-845. The authors next asked what would happen to a slice in which LTP was induced, if following a period of sustained potentiation, low-frequency stimulation was given in order to reverse the established LTP. This time around, there was significant dephosphorylation of Ser-831 (the CaMKII region) and no changes in Ser-845 phosphorylation (the PKA region). This shows that low-frequency stimulation can result in either LTD or reversal of LTP i.e., depotentiation, and the former is associated with dephosphorylation of Ser-845, and the latter with dephosphorylation of Ser-831. Therefore, depending on the recent history of activity of the synapse the GluR1 subunit undergoes dephosphorylation on different sites. The converse was also found to be true, reversal of LTD produced no change in phosphorylation of Ser-831, but increased phosphorylation of Ser-845.

The conclusion that can be drawn from this vast body of evidence is that CaMKII is ideally located, and possesses the intrinsic properties (i.e., its ability to autophosphorylate), that would enable it to play a critical role in the early changes associated with LTP. The fact that CaMKII phosphorylates the receptors that are critically involved in the expression of LTP, in a manner that is regulated by various levels of phosphorylation and dephosphorylation on specific sites adds a further level of complexity. The fact that α CaMKII messenger RNA is distributed in dendrites and cell bodies, (Burgin et al., 1990) places it in a good position to modulate synaptic function. The ability of CaMKII to remain active long after the termination of the initial triggering signal may allow for various downstream, and/or parallel pathways (such as PKA and PKC dependent pathways) to participate in the early expression of LTP or vice versa. Subsequent maintenance of LTP in the later stages may then be facilitated by the translation of CaMKII mRNA to replace, or provide subsistence to degraded proteins at individual synapses. The role that CaMKII may play in the insertion of receptors into the synapse is interesting in light of the suggestion made by Luscher et al., (2000) that increases in the number of synapses with perforated post-synaptic densities observed after LTP may be

a morphological correlate of the mechanisms of receptor trafficking. Further discussion of how this relates to the concept of 'synaptic tagging' follows in Section 1.11.4.

1.9.11 cAMP-dependent protein kinase A (PKA) and E-LTP.

Like PKC and CaMKII, PKA consists of regulatory and catalytic subunits. The mammalian PKA family includes four regulatory subunits; two of each type (Ri α , Ri β , RII α , RII β) and two catalytic subunits (C α and C β), each encoded by a specific gene. Again, there seems to be some confusion as the exact role of PKA in LTP. This controversy again is based on the question of the possible role it might play in induction or expression of LTP.

In a series of experiments Chetkovich et al., (1991), Chetkovich and Sweatt, (1993) found that levels of cyclic AMP, the second messenger activator of PKA, increase immediately after the induction of LTP, and that this increase is followed by a return to basal levels during the subsequent 10 – 20 min. However, application of cell permeant inhibitors of PKA have been shown to only block the late, maintenance phase of LTP, i.e., late-LTP (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994). In these experiments, the PKA inhibitor was present during the tetanus, but LTP was seen to last for at least 3 hr before returning to baseline levels. Additionally, application of the PKA inhibitor KT 5720, 15 min after the tetanus had no effect on the level of the established potentiation (Huang and Kandel, 1994).

Experiments by Roberson and Sweatt, (1996), suggest one possible answer. They attempted to determine whether or not the initial elevation in cAMP produces persistent activation of PKA that is crucial to late-LTP, or whether it exerts its effects by causing a transient elevation of PKA which triggers a cascade of other intracellular events downstream of PKA. They found that multiple high frequency trains used to induce late-LTP produced a significant activation of PKA that began 2 minutes after stimulation and remained elevated for 10 minutes. The activity of PKA was not significantly altered at either 45 minutes or 3½ to 5 hours suggesting that PKA does not remain persistently active during either early- or late-LTP. So the time course of activation of PKA mirrors the change in cAMP concentration. PKA must play a part in late-LTP by mediating other mechanisms that contribute to the prolonged potentiation. Evidence supportive of such a mechanism, and further discussion of PKA, will be postponed until later sections' as we shall now turn to look at late-LTP.



1. 10 Late-LTP.

“LTP has an initial phase (I-LTP, also known as short-term potentiation) lasting approximately 30 min that is insensitive to most protein kinase inhibitors, a protein kinase-dependent early phase (E-LTP) that lasts until 3-4 h after induction, and an ensuing late phase (L-LTP) distinguished by a requirement for RNA and protein synthesis at the time of the inducing stimulus.” from Roberson and Sweatt, (1996). In the following sections, the discussion will focus on the role of protein, and possibly mRNA, synthesis in maintaining L-LTP. There is some evidence that certain second messenger pathways are crucially involved in the maintenance phase of LTP, and also that certain neuromodulatory inputs may have an important role in L-LTP. Activation of transcription factors has been shown to occur as a result of both the induction of L-LTP, and the storage of memories for long periods, so the evidence in favour of this is also addressed.

1.10.1 Protein synthesis and late-LTP.

Hippocampal LTP can persist for weeks (Bliss and Gardner-Medwin, 1973b), and months, even as long as 1 year in awake freely moving animals (Abraham et al., 2002). Previous sections have discussed how the storage of long-term memories seems to be dependent upon protein synthesis at some time before, or just after learning some new information. One of the first experiments to address the possible role of protein synthesis in the maintenance of LTP for long, but not short periods, was that by Krug et al., (1984). They found that in the dentate gyrus of freely moving rats, intraventricular injection of the protein synthesis inhibitor anisomycin 15 min prior to tetanisation, had no effect on the induction of LTP, but did cause a reduction after 5 – 6 hr. LTP in control animals remained elevated for up to 7 days. Similar results have been reported in anaesthetised rats (Otani and Abraham, 1989; Otani et al., 1989). Injections of anisomycin 15 min post-tetanus caused no effect on L-LTP in these experiments, further, an injection 4 hr prior to the tetanus had no greater an effect on LTP than when applied 1 hr before tetanisation (Otani et al., 1989). This suggests that the effect of protein synthesis inhibition on L-LTP is not due to the depletion of existing levels of proteins by anisomycin, but rather L-LTP is blocked due to the inhibition of synthesis of new proteins from pre-existing mRNA.

1.10.2 Translational control in neurons.

Despite widespread evidence that translation is necessary for L-LTP, relatively little is known about how synaptic activity regulates translational activation and repression. Protein synthesis is regulated in many instances at the initiation stage i.e., the stage during which a ribosome is recruited to the 5' end of an mRNA, and positioned at a start codon (for review see Mathews et al., 2000). Eukaryotic ribosome's do not have the ability to locate and bind to the 5' end of mRNA; they must rely on a number of translation initiation factors to guide them there.

Protein translation is controlled by various translation initiation factors, and recent studies have characterised a rapamycin-sensitive translational signaling pathway that regulates the translation of a specific subclass of mRNAs in mammalian cells (Brown and Schreiber, 1996). Activation of this pathway (known as mammalian target of rapamycin, mTOR), modulates the activity of several translation regulatory factors. Activation of mTOR can contribute to translational initiation by phosphorylating proteins that bind eukaryotic initiation factor-4E (eEIF-4E). These proteins are known as eEIF-4E binding proteins (4E-BPs). The association of 4E-BPs with eEIF-4E inhibits the ability of eEIF-4E to associate with eEIF-4G and initiate translation. The phosphorylation of 4E-BPs by mTOR, or other kinases, results in their dissociation from eEIF-4E and a subsequent initiation of translation (Beretta et al., 1996; Brunn et al., 1997).

Very recently, Tang et al., (2002) addressed the potential involvement of the rapamycin-sensitive pathway in L-LTP. Western blot analyses confirmed that mTOR and its various downstream targets (i.e., eEIF-4E, 4E-BPs) are present in hippocampal lysates, and fluorescent immunostaining in hippocampal slices confirmed the presence of these proteins within the CA1 region (both in cell bodies and dendrites). In the presence of rapamycin, LTP was induced, but the magnitude of the potentiation was significantly less after 2 – 3 hr, compared to untreated control slices.

1.10.3 mRNA transcription and late-LTP.

Although the proteins involved in the maintenance of L-LTP may be translated from mRNA already present at, or around, the time of L-LTP induction, transcription of mRNA may also play some part in the maintenance of L-LTP. Daniels, (1971), showed that short-term memory (< 3 hr) was unaffected when rats had been given an intrahippocampal injection of

the transcriptional inhibitor actinomycin D, 4 hr before training. However, the animals long-term memory (> 4 hr) was greatly impaired.

Studies on LTP *in vivo* by Otani and Abraham, (1989), Otani et al., (1989) found that either intraventricular injection of actinomycin D just prior to tetanisation in anaesthetised rats, or focal injections into the dentate gyrus 1 hr prior to tetanisation did not affect the maintenance of LTP for up to 3 hr post-tetanus, whereas anisomycin did block L-LTP during this time. The authors suggest that the proteins necessary for the maintenance of LTP are synthesised from pre-existing mRNA. However, in the freely moving animal Frey et al., (1996) found that intraventricular injection of actinomycin D, 2 hr before tetanisation blocked L-LTP and the earliest time at which this difference was observed was 5 hr post-tetanus. In the same report, LTP *in vitro* was blocked by actinomycin D, only if the drug was applied before the tetanus. If the drug was applied just after the last of three high frequency trains, no effect on L-LTP was seen for up to 8 hr post-tetanus. A similar finding was reported by Nguyen et al., (1994) who found that when they applied actinomycin D for 2 hours immediately after 3 high frequency trains the mean EPSP slope was significantly reduced, an effect that was evident about 2 - 3 hours after LTP induction. However if they delayed application of the drug to 2 hours after the first tetanus then no reduction in the EPSP was observed 4.5 hours after LTP induction. Therefore, mRNA transcription may be important for the induction of the later stages of LTP, possibly by providing new mRNA that can be subsequently translated into the proteins involved in the long-term maintenance of L-LTP. There is substantial evidence that certain transcription factors are activated by stimuli that induce L-LTP, and as a result of long-term storage of memory. The evidence pointing to the role of CREB in L-LTP and long-term memory will be discussed later.

1.10.4 Second messenger systems and late-LTP.

In previous section, evidence was reviewed that focussed on the role that proteins such as PKC and CaMKII may play in either the induction or expression of E-LTP. The point was made that although both of these pathways appear to be involved in the expression mechanisms underlying the initial expression of LTP, they may also play a role in later stages by interactions that occur at the synapse in terms of expression of forms of these proteins that are active long after the end of the signal that initially triggered them. One possible mechanism is that these proteins interact together to alter the phosphorylation/dephosphorylation state of various substrates. There is considerable evidence

that the cAMP dependent protein kinase A (PKA) works like this, and may play a key role also in the downstream signalling pathways involved in the maintenance of L-LTP. Some of the earliest work suggesting that the cAMP pathway was involved in L-LTP in the hippocampal slice came from experiments looking at dopamine receptor activation.

1.10.5 Dopamine and late-LTP.

The D1 dopamine receptor subtype is positively coupled to adenylyl cyclase (Sunahara et al., 1991). Frey et al., (1990) found that an antagonist of the D₂-dopamine receptor subtype, domperidone (1 μ M), had no effect on baseline synaptic transmission. However when applied 15 minutes before LTP induction, both the population spike and the EPSP potentiation were significantly reduced when compared to slices that were stimulated without the drug present. The earliest effect of the antagonist was evident approximately 4 hours after the last tetanus, and no effects on earlier stages of LTP were observed. This blockade of the later stages of LTP was reversed if the dopamine agonist apomorphine (1 μ M) was applied in conjunction with domperidone, such that there was no significant difference between these slices and those that were similarly tetanised but without any drugs present. Flupenthixol, an antagonist of the D₁-receptor subtype, (1 μ M) also significantly attenuated the prolonged potentiation. Release of radiolabelled dopamine from hippocampal slices was also found to be significantly increased following a train of 100 pulses at 100 Hz, when compared to a control protocol of 4 pulses at 0.33 Hz.

Frey et al., (1991) carried out similar experiments with the selective D₁/D₅-receptor antagonist SCH 23390 (0.1 μ M) and discovered that application of this drug 15 minutes before three-fold tetanisation elicited a short-lasting potentiation of both the population spike and the EPSP that was significantly different from untreated tetanised controls. The decrease was significant 30 minutes after the last tetanus and continued to decline up to the fifth hour. Further discussion of the role that dopamine plays in L-LTP shall be delayed until Chapters' 6 and 7 as the experiments reported in these chapters focus on dopamine, L-LTP and long-term memory.

1.10.6 cAMP dependent protein kinase A (PKA) and late-LTP.

cAMP activates PKA by dissociating its catalytic from its regulatory subunits. PKA has been suggested to play a role in late-LTP from a number of experiments. Frey et al., (1993)

examined the effects on late-LTP of inhibiting PKA with a membrane-permeable cAMP analog, Rp-cAMPS (100 μ M). When this competitive inhibitor was applied to slices 15 minutes before inducing LTP, it produced a small effect on the potentiation after 30 to 60 minutes (early-LTP), but completely abolished the potentiation of both the EPSP and the population spike observed at later stages, i.e. 7½ hours later. This effect was specific to the drug's effect on PKA as application of Sp-cAMPS a membrane-permeable activator of PKA resulted in a delayed potentiation that persisted for 3 hours, and occluded subsequent induction of LTP by tetanic stimulation (the converse occlusion of subsequent Sp-cAMPS induced LTP by prior tetanic stimulation was also observed). This suggests that Sp-cAMPS induced potentiation shares similar mechanisms to high frequency stimulation induced late-LTP. Similar results have been reported by others (Bolshakov et al., 1997; Nguyen and Kandel, 1997). Application of PKA inhibitors after tetanisation typically has been found not to inhibit L-LTP (Huang and Kandel, 1994).

1.10.6. Genetic demonstration for a role of PKA in L-LTP.

As was mentioned before, PKA is made up of regulatory and catalytic subunits. There are four different regulatory subunits (RI α , RI β , RII α , RII β) and two catalytic subunits (C α , C β) in the PKA family. Qi et al., (1996) produced mice with a mutation in the C β ₁, an isoform of the C β subunit. These mice showed a deficit in the later stages of LTP induced with multiple high frequency trains. The mutants showed a normal early phase, but the potentiation continuously decayed over time, resulting in late-LTP that was significantly less than wild-type mice did at 3 hours.

Abel et al., (1997) reduced PKA activity by using a dominant negative form of the RI α regulatory subunit of PKA, R(AB). This was under the control of the CaMKII α promoter. These transgenic animals showed a 50% reduction in basal PKA activity and a 25% reduction in cAMP-induced activity. There were no differences between wild type and mutant animals in terms of pre-synaptic function or post-tetanic potentiation. The mutation appears to affect the more long-lasting potentiation induced by multiple trains of stimuli as these transgenic animals showed a decremental form of late-LTP that was first apparent 70 min after 4-fold tetanisation, and which continued to decline up to the end of the experiment (3 hr). These animals also were impaired in a contextual fear conditioning paradigm, when they were tested 24 hr after being exposed to the conditioning apparatus, but not when tested 1 hr later.

1.10.7 Mitogen-activated protein kinases (MAPK) and L-LTP.

In an earlier section, the involvement of MAPK in the induction of LTP was introduced. One of the experiments discussed in that section raised the possibility that this pathway may regulate the phosphorylation of CaMKII in a translation dependent manner (Giovannini et al., 2001). There is some evidence that this pathway may be a point of convergence for PKA, PKC and CaMKII (Impey et al., 1998b; Roberson et al., 1999). Additionally, in cell lines, MAPK has been found to translocate to the nucleus once activated, where it regulates the transcriptional activity of a number of immediate early genes (see Treisman, 1996 for review).

In the discussion of second messenger systems in LTP, the point was raised concerning the timing of drug application and how this may affect distinguishing between effects on induction and maintenance of LTP. A number of experiments where MAPK inhibitors are applied for long periods before a tetanus have suggested that this pathway is involved in the induction of LTP (English and Sweatt, 1996, 1997; Liu et al., 1999). In an attempt to distinguish between effects on early- vs. late-LTP, Rosenblum et al., (2002) applied the MEK inhibitor PD98059 both 40 min before, and immediately after the tetanus. When applied before the tetanus, there was a significant reduction in the drug treated slices 20 min after the tetanus, and this declined further during the subsequent 4 hr. When the drug was applied immediately (2 min) after the tetanus no difference was observed 60 min post-tetanus, however 4 hr after the tetanus the drug treated slices no longer showed any potentiation at this time.

How might the MAPK pathway be involved in both E-LTP and L-LTP? Evidence suggests that some kinases, such as PKA, may have a 'gating' function in LTP by inhibiting protein phosphatases, thereby allowing the persistent activation of other proteins, possibly PKC or CaMKII (Blitzer et al., 1995). Roberson et al., (1999) suggest that MAPK is coupled to both PKA and PKC. In hippocampal slices activation of PKA or PKC resulted in phosphorylation of MAPK in CA1 and in both cases this was blocked by PD098059. They then went on to investigate whether the activation of the neuromodulatory transmitter systems that are known to activate these pathways (i.e., dopamine and β -adrenergic receptors for PKA; muscarinic acetylcholine and metabotropic glutamate receptors for PKC) also result in MAPK phosphorylation. Significant phosphorylation of MAPK was seen in response to specific agonists of these receptors, and the corresponding receptor antagonists blocked this effect. To

examine whether the coupling between these receptor mediated pathways and MAPK occurs via the activation of PKA, the experiment was repeated with the PKA inhibitor H89. In this condition, H89 blocked the phosphorylation of MAPK that was seen after activation of either dopaminergic or adrenergic receptors, suggesting that PKA is activated upstream of MAPK.

Therefore, MAPK may regulate the way in which PKA ‘gates’ the induction of LTP. One possible mechanism that may explain this is if MAPK activation modulates different ion channels (i.e., voltage dependent K^+ or Ca^{2+} channels) in the postsynaptic receptor. Enhanced depolarisation may allow for increases in intracellular Ca^{2+} , which may then trigger protein kinases such as PKA, PKC, and CaMKII. Impey et al., (1998b) have also suggested that the MAPK pathway is a point of convergence of multiple Ca^{2+} signalling pathways involved in the generation of L-LTP.

1.10.8 The ‘gating’ hypothesis.

In earlier sections the ‘apparent’ discrepancy between the effects that PKA inhibitors have on only L-LTP and the biochemical evidence showing that PKA is only transiently activated following LTP induction was discussed. The experiments of Roberson and Sweatt, (1996), suggested that PKA must play a part in late-LTP by mediating other mechanisms that contribute to the prolonged potentiation as activation of PKA was found to occur only transiently after LTP induction. Huang and Kandel, (1994) found that the PKA inhibitor KT5720 did not affect LTP when it was applied to the slice 15 minutes after the last of three tetani. So the time course of activation of PKA mirrors the change in cAMP concentration.

It has been suggested that PKA may play a ‘gating’ function in the induction of LTP by inhibiting protein phosphatases, thereby allowing the persistent activation of other proteins, possibly PKC or CaMKII. The first experimental evidence which suggests such a gating role for PKA came from Blitzer et al., (1995). The pyramidal cells of the hippocampus have a slow Ca^{2+} -activated potassium current. Activation of the NMDA receptor causes the influx of Ca^{2+} through its own and separate voltage-gated Ca^{2+} channels, that together trigger the opening of potassium channels producing a prolonged hyperpolarization. Many transmitters modulate this current without producing any effect on the resting membrane potential. One such transmitter is norepinephrine. It inhibits this slow after-hyperpolarization (AHP); therefore excitatory inputs that might normally produce only a few action potentials before being cut off by the after-hyperpolarizing effect now evoke a sustained train of impulses.

Blitzer et al., (1995) used whole-cell recordings to see whether changes in the AHP could serve as an assay for elevations in cAMP. Whole-cell recordings were made of CA1 cells after the delivery of a single high frequency stimulus (HFS) to the Schaffer-collaterals. This produced a suppression of the AHP, which was maximal 1-2 minutes after the stimulus and recovered within 15 minutes. PKA appears to play an important part in this effect as when the pipette contained either excess regulatory subunits or Rp-cAMPS (200 μ M), the suppression of the AHP that follows HFS was blocked. With intracellular recordings, three trains, of the type used to elicit L-LTP, in the presence of the phosphodiesterase inhibitor IBMX (1 mM) were required to produce the same suppression of the AHP seen in the whole-cell mode. Again, when Rp-cAMPS was present in the electrode the HFS-induced suppression of the AHP was blocked.

Blitzer et al., (1995) suggest that PKA acts as a gate. Rather than the cAMP/PKA pathway transmitting the signal for this early stage of LTP, it activates other systems, permitting the signal to flow through the cell. The reason they suggest that this is as follows. Slices were stimulated with 3 trains of HFS in the presence or absence of Rp-cAMPS. When applied to the bath, this inhibitor of PKA had already produced a significant reduction 40 minutes after the last train. Intracellular injection of the drug produced an even more profound effect such that 5 minutes after the last train, the potentiation in the Rp-cAMPS group was no longer significant. This early stage appears to be entirely dependent on PKA. If this PKA dependent pathway is the signal for the downstream effect, then one might expect that the increase in synaptic efficacy that accompanies LTP is directly the result of cAMP/PKA. cAMP is known to enhance the responses mediated by AMPA receptors in cultured hippocampal cells (Greengard et al., 1991). The cAMP/PKA pathway may increase the efficiency of synaptic transmission that accompanies late-LTP. They tested this by applying Sp-cAMPS to slices and looking at what effects it had on the AHP and the amplitude of the intracellular EPSP. If the PKA acts as described above, then one would expect to see an increase in the amplitude of the EPSP after activation of cAMP. Sp-cAMPS, which activates PKA, caused a fast inhibition of the AHP (within 5 minutes). Nonetheless, the EPSP increased only very slightly during the next 60 minutes, indicating that even though Rp-cAMPS completely inhibited LTP in such time, no increase in the EPSP was seen after application of Sp-cAMPS. The mechanism by which this may happen is via inhibition of protein phosphatases by inhibitor 1. Protein phosphatase inhibitors should protect LTP from the inhibitory effect of Rp-cAMPS. When recordings were made with the phosphatase inhibitor microstatin-LR in the recording electrode, it prevented Rp-cAMPS from inhibiting LTP. Therefore, it would appear that the

cAMP pathway is involved, in a specific way, in E-LTP, but the mechanism by which this occurs may also implicate PKA in L-LTP, as PKA may inhibit protein phosphatases and therefore signalling via other pathways may be sustained. Otmakhova et al., (2000), found that by using specific inhibitors of PKA, effects on E-LTP were clearly produced.

1.11 Transcriptional regulation of long-term synaptic and behavioural plasticity.

L-LTP may involve each of these different signalling pathways to different degrees, but there may also be points of convergence between each. What is clear however, is that the synthesis of new proteins is essential for the long-term persistence of this form of plasticity. Transcription and translation are processes predominantly associated with the nucleus (Wenzel et al., 1993), therefore a signal must be relayed from the synapse to the nucleus in order to trigger the synthesis of proteins that are required to stabilise the potentiated state of the activated synapses. The most compelling evidence of how transcription is regulated, and how this is associated with LTP comes from studies involving one particular family transcription factors, namely cAMP-response-element-binding proteins (CREB).

1.11.1 CREB and long-term memory.

Signalling by changes in the intracellular concentration of cAMP may lead to changes in gene expression. This possibility arises as there is a family of transcription factors called cAMP-response-element-binding proteins (CREB), that promote the transcription of genes with cAMP response elements (CREs) in their promoter sequence. Phosphorylation of CREB on Ser 133 can be brought about by a number of protein kinases including PKA, CaMKII, CaMKIV, and MAP kinase-activated protein (see Bito et al., 1997 for a review). Phosphorylated CREB then binds to its co-activator, CREB-binding protein (CBP), which facilitates the unwinding of DNA and interacts with the basal transcriptional machinery (Chrivia et al., 1993; Kwok et al., 1994). As activation of these signalling molecules may result from a wide diversity of signals, a large body of evidence has accumulated that suggests that CREB may be important in a number of cellular responses. CREs lie upstream of a number of genes, including the immediate early genes *c-fos* (Sassone-Corsi et al., 1988) and *zif268* (Sakamoto et al., 1991) and also genes encoding synapsin I (Sauerwald et al.,

1990) a K^+ channel (Mori et al., 1993) BDNF (Shieh et al., 1998; Tao et al., 1998) and the α subunit of CaMKII (Olson et al., 1995).

Some of the original experiments that suggested a link between CREB and synaptic plasticity came from work with *Aplysia*. Both long-term and short-term sensitization of the gill and siphon withdrawal reflex in *Aplysia* involve facilitation of the monosynaptic connections between the sensory and motor neurons. To analyze the relationship between these two forms of synaptic facilitation at the cellular and molecular level, this monosynaptic sensorimotor component of the gill-withdrawal reflex of *Aplysia* can be reconstituted in dissociated cell culture. Whereas one brief application of serotonin produced short-term facilitation in the sensorimotor connection that lasted minutes, five applications over 1.5 hr resulted in long-term facilitation that lasted more than 24 hr (Montarolo et al., 1986). Dash et al., (1990) subsequently demonstrated that inhibition of CREB function by injection of a CRE oligonucleotide into the nucleus of the presynaptic cell blocked long-term, but not short-term facilitation at this synapse. Subsequent work confirmed these findings (Bartsch et al., 1995; Bartsch et al., 1998).

Studies of *Drosophila* also lend support to the notion that CREB is important in long-term memory. Yin et al., (1994, 1995) found that inducible expression of a CREB activator enhanced memory, whereas, expression of a CREB repressor blocked the formation of long-term memory for an associative olfactory learning task. The first series of experiments that addressed whether or not CREB plays a role in long-term memory in mammals was that of Bourtchouladze et al., (1994). They analysed mutant mice that lack both α and δ isoforms of CREB in both behavioural and electrophysiological experiments. These mice were found to be deficient in a cued and a contextual fear-conditioning task when tested 24 hr after training, but not when tested 30 min or 1 hr after training. These animals also displayed a spatial learning deficit (in watermaze performance) even though intensive training was able to rescue this deficit somewhat. LTP was also disrupted in these animals as mutant slices tetanised with a single 100 Hz train showed decremental LTP that returned to pre-tetanus levels over 2 hr.

However, there are complications as only some forms of CREB are deleted in these mice, and both these undisrupted forms, and the CREB-related gene CREM, are upregulated in compensation (Hummler et al., 1994; Blendy et al., 1996). Problems associated with the genetic background of the animals in the Bourtchouladze et al., (1994) study have been suggested as explanations for the deficits seen (Gass et al., 1998). There are also cases where

CREB mutant mice have been found to have no phenotype in either learning or synaptic plasticity (Gass et al., 1998; Rammes et al., 2000).

More recently, Pittenger et al., (2002) generated transgenic mice that express a mutant of human CREB that is a potent dominant-negative inhibitor of all three CREB transcription factors. The expression of the gene was restricted to the dorsal hippocampus (using the α CaMKII promoter) and temporal control was provided by use of the tetracycline-regulated tTA system (Mayford et al., 1996b). These animals showed a deficit in performance of the watermaze during the probe trial, where they spent less time searching in the correct quadrant than did control animals. In contrast to the previous studies, these animals performed normally on both cued and contextual fear conditioning when tested 24 hr after training. The authors suggest that this is due to the fact that the transgene was expressed solely in the dorsal hippocampus; cytotoxic lesions to the dorsal hippocampus was found not to impair contextual conditioning (Frankland et al., 1998). However, others suggest that contextual conditioning is dependent on CRE-mediated transcription in the dorsal hippocampus (Athos et al., 2002). The animals in the Pittenger et al., (2002) experiment were impaired on an object recognition task 24 hr after training, and this deficit could be ‘rescued’ by switching off the gene with doxycycline treatment.

1.11.2 CRE-mediated gene expression and long-term memory.

The experiments discussed above all suggest that CREB activity is important for long-term memory, however, it has yet to be shown that CRE-mediated gene expression is activated during long-term memory formation.

Impey et al., (1998a) used mice transgenic for a CRE-*lacZ* reporter to assess whether memory formation triggers CRE-mediated transcription. They trained animals in a contextual fear conditioning apparatus and learning was measured 8 hr after training. Mice that were contextually conditioned (but not mice that received no shock, or unpaired shocked mice) showed strong contextual conditioning. Only this group showed significant increases in CRE-mediated transcription in both CA1 and CA3 neurons. This increase in CRE-mediated transcription was associated with increases in phosphorylated CREB at Ser 133. Subsequent experiments by Taubenfeld et al., (1999) showed that lesions of the fornix (the fibre bundle that connects the hippocampus to the septum and hypothalamus) specifically disrupt both

long-term memory for an inhibitory avoidance task, and phosphorylation of CREB in the hippocampus.

1.11.3 CRE-mediated gene expression and synaptic plasticity.

Allied to the analysis of the role that CRE-mediated gene expression via phosphorylation of CREB in long-term memory, many experiments have investigated whether synaptic activity leading to LTP activates the CREB pathway. One of the first experiments of this nature was performed by Impey et al., (1996). They generated mice transgenic for a CRE- β -galactosidase reporter construct to see if stimuli that generate L-LTP in hippocampal slices are associated with changes in CRE-mediated transcription. They used different tetanus protocols to differentiate between decremental, or E-LTP, induced by a single 100 Hz train, and L-LTP induced by three 100 Hz trains. No changes in CRE-mediated gene expression were found to occur following E-LTP induction 4 hr after the tetanus, whereas L-LTP was associated with significant increases in β -galactosidase staining, that was detectable 2 hr post-tetanus and reached a maximum at 6 hr. Similar increases in CRE expression were seen after treatment with the adenylyl cyclase activator forskolin, or the D1/D5 dopamine agonist SKF38393, suggesting that recruitment of the cAMP pathway is involved in the upregulation of CRE-dependent transcription. Allied to this are the findings that PKA inhibitors specifically blocked the maintenance of L-LTP and increases in CRE expression.

One unusual finding was that application of NMDA receptor antagonists did not affect L-LTP, but completely blocked E-LTP. CRE-mediated gene expression was also similarly unaffected by the presence of NMDA antagonists during L-LTP induction (Impey et al., 1996). However, blockade of voltage-gated calcium channels inhibited L-LTP after 4 hr, and reduced the expression of CRE. Previously Reymann et al., (1989) showed that both E-LTP and L-LTP were dependent on NMDA receptor activation, therefore it is not clear why this discrepancy was found.

1.11.4 What signalling cascades are involved in CRE-mediated gene expression?

Experiments by Deisseroth et al., (1996) attempted to identify the conditions by which CREB is phosphorylated in response to stimulation in more detail, and to determine what signalling cascades are involved in CREB phosphorylation. In cultured hippocampal neurons, high frequency tetanisation produced robust CREB phosphorylation that was seen within 30 s. In

the presence of glutamate receptor antagonists (AP5 and CNQX), no changes in CREB phosphorylation were seen. Therefore, phosphorylation of CREB appears to be dependent upon synaptic stimulation and not action potential firing. When Ca^{2+} transients were monitored with fluo-3 Ca^{2+} imaging, increases in Ca^{2+} were seen in the cytoplasm and nucleus in response to high frequency stimulation even when AP5 and CNQX were present, i.e., a condition in which CREB phosphorylation was blocked. Increases in nuclear Ca^{2+} do not seem to be sufficient to trigger CREB phosphorylation. Deisseroth et al., (1996) suggest that the key synaptic signal for CREB phosphorylation in the nucleus may involve activation of calmodulin. Application of calmidazolium, a membrane permeable calmodulin inhibitor, completely blocked CREB phosphorylation in response to LTP inducing synaptic stimulation. Previously, Gonzalez and Montminy, (1989) showed that CREB was phosphorylated by cAMP on Ser-133. However, Deisseroth et al., (1996) suggest that the mechanism by which the binding of Ca^{2+} by calmodulin leads to an increase in CREB phosphorylation is due to a direct activation of protein kinases, possibly CaMKII, as several PKA inhibitors did not block nuclear CREB phosphorylation in response to LTP (Dash et al., 1991), whereas Impey et al., (1996) found that the PKA pathway was involved in CREB phosphorylation in hippocampal slices. On the other hand, the Ca^{2+} /calmodulin-dependent kinase inhibitor KN-62, completely blocked CREB phosphorylation (Deisseroth et al., 1996).

Subsequent experiments by the same group suggested that calmodulin kinases (such as CaMK II and IV) are the key regulators of CREB phosphorylation (Bito et al., 1996). These experiments showed that a brief depolarisation of cultured hippocampal neurons, which induces CREB phosphorylation, also maximally activates CaMKIV. They also found that once CREB is phosphorylated, the maintenance of this state is regulated by protein phosphatase 1. The decline in CREB phosphorylation also seems to be regulated by calcineurin (CaN), as inhibition of CaN retarded the decay of phosphorylated CREB. CaN may act by triggering protein phosphatase 1, by dephosphorylating inhibitor 1, thereby inhibiting CaMKIV or its activators. Rapid activity-dependent translocation of calmodulin to the nucleus of cultured hippocampal neurons has been suggested to be the mechanism by which the signalling from the synapse to the nucleus where it may phosphorylate nuclear CaM kinases, leading to CREB phosphorylation (Deisseroth et al., 1998).

In order for CRE-mediated gene expression to occur, CREB must be phosphorylated. When phosphorylated on Ser-133 by either cAMP (Gonzalez and Montminy, 1989), or Ca^{2+} /calmodulin dependent protein kinases (CaMKII) (Dash et al., 1991; Sheng et al., 1991) it

promotes transcription from the CRE sequence. Impey et al., (1996) found that both E-LTP and L-LTP were associated with increases in phosphorylation of CREB on Ser-133. As CRE-mediated gene expression did not occur after E-LTP, this finding is consistent with the finding that phosphorylation at Ser-133 is not sufficient for CRE-mediated gene expression (Ginty et al., 1994; Brindle et al., 1995; Thompson et al., 1995). However, as the experiments of Bito et al., (1996) highlight, only prolonged CREB phosphorylation correlates with CRE-mediated gene expression, therefore additional signalling cascades may be involved in the phosphorylation of CREB in order to produce transcription. A second regulatory event is critical for CRE-mediated gene expression, that is, activity of the CREB binding protein (CBP), (Chawla et al., 1998; Hardingham et al., 1999; Hu et al., 1999).

Impey et al., (1998b) have suggested that activation of a Ras/mitogen-activated protein kinase (MAPK) or extracellular signal-regulated protein kinase (ERK)-mediated pathway modulated by PKA is important in CREB phosphorylation. Recent experiments by Wu et al., (2001) suggest that there is some convergence in the signalling pathways between the CaMK-dependent pathway and the Ras/MAPK pathway. In cultured neurons that were depolarised by exposure to K⁺, they found that there were two periods of intense CREB phosphorylation. The first occurs rapidly and was inhibited by the CaMK inhibitor KN-93 and the MAPK inhibitor PD98059 inhibited the second more delayed rise in CREB phosphorylation. Similar effects on the delayed, but not the early rise in CREB phosphorylation were seen in neurons co-transfected with dominant negative Ras. Additionally, a milder depolarising stimulus (which still produced substantial Ca²⁺ influx), did not result in significant activation of ERK, and the delayed peak in CREB phosphorylation was notably absent, which suggests that substantially more Ca²⁺ is required to activate the MAPK pathway leading to CREB phosphorylation than that required for the CaMK pathway. This is suggestive of another mechanism whereby the intensity of stimulation may be transmitted downstream to differential modulation of transcription.

However, there is some controversy concerning the nature of the Ca²⁺ signal in CREB phosphorylation. Although increases in Ca²⁺ appear to be necessary for the activation of all the pathways implicated in CREB phosphorylation (cAMP, PKA, CaMK, ERK/MAPK), whether changes in Ca²⁺ alone are sufficient is not clear. The involvement of both NMDA receptors and L-type Ca²⁺ channels appears to be crucial, and the signal that mediates CREB phosphorylation has been suggested to be carried out by CaM (Deisseroth et al., 1996; Deisseroth et al., 1998; Mermelstein et al., 2001). This translocation of CaM to the nucleus

may occur in a complex with another protein. Mermelstein et al., (2001), suggest that this protein is one of the Ca^{2+} /calmodulin dependent kinases (CaMK) as the inhibitor KN-93 completely blocked CREB phosphorylation and CaM translocation. Such translocation may enable the fast phosphorylation of CREB that appears to be dependent on CaM kinases (Wu et al., 2001).

Although all this evidence highlights the importance of Ca^{2+} entry at local synaptic sites in order to trigger the plethora of signalling cascades that appear to be involved in CREB phosphorylation, (via translocation of CaM to the nucleus Deisseroth et al., 1996; Deisseroth et al., 1998; Mermelstein et al., 2001) others have suggested that elevations in nuclear Ca^{2+} may be sufficient in itself to trigger CREB phosphorylation (Hardingham et al., 2001). Hardingham et al., (2001) suggest that calcium itself, rather than a Ca^{2+} /calmodulin complex is the messenger that couples synaptic activity to nuclear transcription, as they found no evidence in hippocampal slices at least for CaM translocation to the nucleus. Deisseroth et al., (1996, 1998) used cultured neurons. Hardingham et al., (2001) suggest that calcium release from intracellular stores amplifies the synaptic signal in the form of a 'regenerative wave' that is propagated to the nucleus.

One hypothetical mechanism may involve both systems. The fast, early peak in CREB phosphorylation may involve the CaMK pathway, and translocation of CaM to the nucleus, whereas the delayed MAPK dependent pathway may be triggered more slowly by prolonged stimulation which causes significant elevations in nuclear Ca^{2+} . However, it is not clear therefore why Deisseroth et al., (1996) found that even though nuclear Ca^{2+} concentrations were significantly elevated when synaptic input was absent, no changes in phosphorylation of CREB were seen, unless the MAPK pathway is also dependent, in part, on activation of the CaMK pathway (as has been suggested by Wu et al., (2001).

1.11.5 Does CREB phosphorylation occur in response to LTP in vivo?

All these experiments discussed so far have involved CREB phosphorylation in a variety of *in vitro* preparations, but does CREB phosphorylation also occur *in vivo* in response to the induction of LTP? Schulz et al., (1999) monitored CREB activation with a phosphospecific antibody in animals chronically implanted for LTP recordings in the dentate gyrus. Phosphorylation of CREB was seen as soon as 5 min after tetanisation in the granule cell layer of the ipsilateral dentate and this extended to the contralateral dentate gyrus over the

following 30 min. One hour after stimulation CREB phosphorylation had completely disappeared, however a second wave of phosphorylation was seen beginning 2 hr after the tetanus and this lasted for at least 24 hr. It is possible that the 'early' peak is dependent upon a CaM kinase pathway, with the ERK/MAPK pathway being involved in the 'late' peak as suggested previously (Wu et al., 2001). This pattern of CREB phosphorylation was seen after a tetanus that produced a non-decremental form of potentiation that lasted at least 6 hr (in some cases, LTP persisted for up to 24 hr). With a 'weak' tetanus that resulted in decremental LTP, only a small transient elevation in CREB phosphorylation was seen within 2 hr, with no phosphorylation after 6 hr.

1.12 Signalling from the synapse to the nucleus: How do 'local' synaptic signals interact with 'diffuse' nuclear messages?

L-LTP exhibits a requirement for both transcription and translation. Since these are primarily somatic events (Wenzel et al., 1993), and individual synapses are capable of being modified independently of each other the problem of how mRNAs or proteins selectively target potentiated synapses arises. In the absence of a mechanism for selectivity, the induction of L-LTP at one set of synapses might result in the heterosynaptic development of potentiation at neighbouring synapses. There are at least three possible solutions to this problem that have been termed, the 'mail' hypothesis, the 'local synthesis' hypothesis, and the 'synaptic tag' hypothesis (reviewed by Frey and Morris, 1998b; Martin and Kosik, 2002).

1.12.1 The 'Mail' hypothesis.

According to the 'mail hypothesis' (schematically illustrated in Figure 1.2 a-d), synapse-specificity is preserved during the maintenance of L-LTP due to the specific targeting of proteins to only those synapses undergoing LTP. There are two key features of this hypothesis; proteins are somehow selectively targeted to recently activated synapses, and as a consequence, the persistence of LTP is determined at the time of LTP induction. There is not much known how such specific targeting might be accomplished during synaptic plasticity, and it has also been questioned whether in fact such a mechanism is likely to occur at all (Frey and Morris, 1998b). The main reason for this is that the initial apparent simplicity of the idea masks the inherent complexity of the mechanism that is required to support it. An individual CA1 pyramidal cell may possess in excess of 10,000 individual synapses and it is

unlikely that the nucleus can somehow ‘remember’ where to traffic proteins such that individual synapses are modulated independently of each other.

There is no direct evidence that this process actually occurs within neurons during synaptic plasticity. However, movement of mRNA from the cell body into dendrites has been observed. Davis et al., (1987, 1990) using [³H]-uridine pulse-chase experiments, showed that the [³H]-uridine labelled mRNA moved out of the cell body and along dendrites in cultured hippocampal neurons. This movement of mRNA was subsequently found to occur following electrophysiological stimulation, as high frequency activation of the perforant path projections to the dentate gyrus caused newly synthesised mRNA for the immediate-early gene (IEG) *Arc* to localise selectively in activated dendritic segments (Steward et al., 1998). Newly synthesised *Arc* protein also accumulated in the portion of the dendrite that had been synaptically activated. The targeting of *Arc* mRNA was not disrupted by locally inhibiting protein synthesis, indicating that the signals for mRNA localisation reside in the mRNA itself. However, this does not indicate that specific trafficking of these proteins to individual dendrites undergoing plasticity takes place. This localisation could equally be due to local translation of the proteins synthesised within the dendrite (i.e., the ‘local synthesis hypothesis’) or due to the capture of these proteins by recently active synapses (i.e., the ‘synaptic tag’ hypothesis).

1.12.2 The ‘local synthesis’ hypothesis.

The ‘local synthesis’ hypothesis relies on the presence of all the necessary machinery for protein synthesis near or possibly within an individual dendritic spine (see Figure 1.2 e-g). Synaptic signals generated in the dendrite are thought to be coupled to protein synthesis machinery in dendrites resulting in the local, synapse-specific production of proteins required for maintaining L-LTP. The discovery that polyribosomes and various mRNAs could be detected within spines fuelled this idea (Steward and Levy, 1982). And several observations suggest that proteins can be made locally within dendrites. Feig and Lipton, (1993) revealed that in the CA1, when repetitive synaptic activity was paired with application of the cholinergic agonist carbachol, protein synthesis within dendrites (assayed with [³H]-leucine incorporation) was increased, although this was not associated with sustained increases in synaptic plasticity. One experiment that has stood in the way of the ‘local synthesis’ hypothesis is that of Frey et al., (1989b). In this experiment the dendritic region of the CA1 subfield was recorded after isolating the apical dendrites from their somata by microsurgical

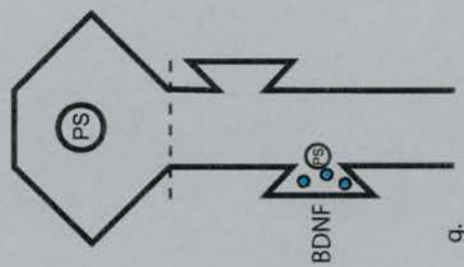
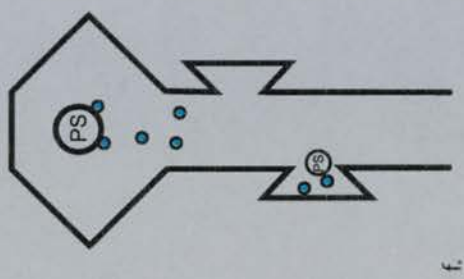
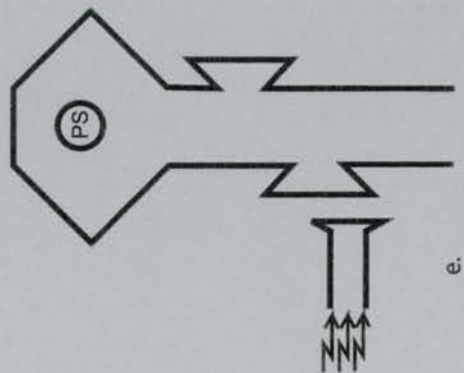
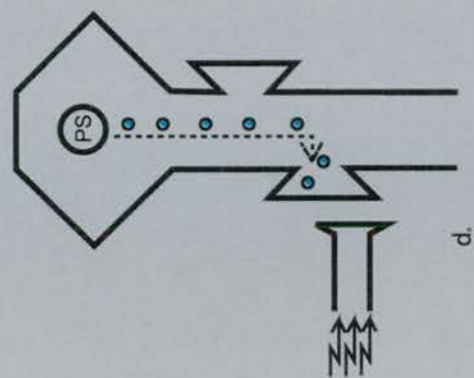
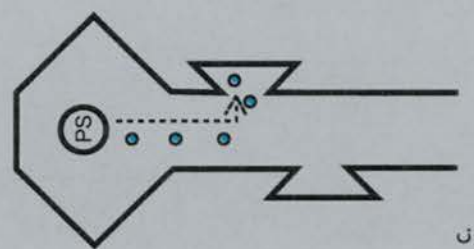
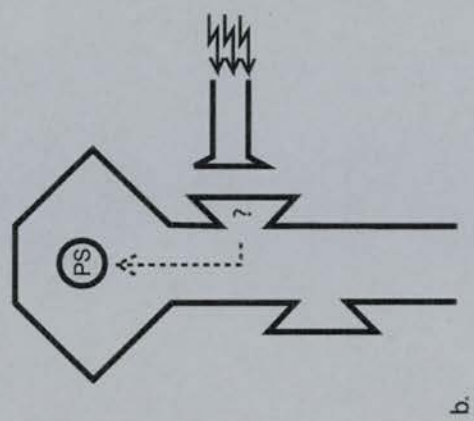
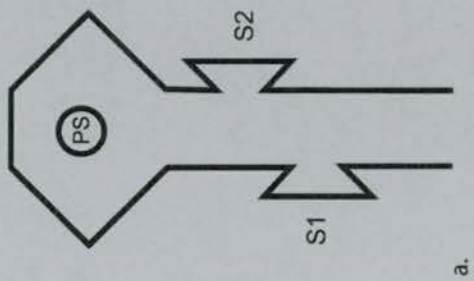
cuts through the proximal stratum radiatum. These experiments revealed that in contrast to intact slices which, after tetanic stimulation of the Schaffer collaterals, showed LTP for 8-10 hr post-tetanus, LTP declined in the isolated dendrites to baseline values after 3 hr. However, they did not address whether LTP in these isolated dendrites is affected by application of protein synthesis inhibitors (see Kang and Schuman, 1996). Evidence was reviewed earlier suggesting that mRNA transcription may be important at later stages in the maintenance of L-LTP; by severing the dendrites from the cell bodies the trafficking of mRNAs from the cell body that are important in the long-term maintenance of L-LTP will have been compromised in this experiment.

However, there may still be some requirement for trafficking of mRNA from the soma to the synapse where these mRNAs are locally regulated by the polyribosomes present within dendrites. The local regulation of mRNAs at individual synapses may still require the presence of a local signal that regulates the translation of these dendritically localised proteins.

Figure 1.2 Solutions to the synapse-to-nucleus navigational problem.

a – d: The ‘mail hypothesis’ proposes that the temporal persistence of LTP at any given input pathway (a) is regulated by the activation (b) of a signal sent to the nucleus that subsequently triggers the synthesis of proteins (c) that are directed back to only those synapses activated by the tetanus. The persistence of LTP is therefore determined at the time of induction (d).

e – h: The ‘local synthesis’ hypothesis is based on the idea that the induction of LTP (e) is associated with the activation of local, dendritic protein synthesis (f) at or close to the activated synapses thereby by-passing the need for proteins to be sent from the nucleus at all. The most convincing evidence for this comes from experiments where the neurotrophin BDNF has been shown to induce protein synthesis dependent potentiation in dendrites isolated from their cell bodies (g).



1.12.3 The ‘synaptic tag’ hypothesis.

An alternative mechanism for maintaining synapse-specificity without the need for any complicated selective targeting of proteins to individual synapses was first proposed by Frey and Morris, (1997). The synaptic tag hypothesis proposes that the persistence of LTP is mediated by the intersection of two dissociable events (see Figure 1.3). The first involves the generation of a local synaptic tag at specific synapses activated by the induction of LTP. The second involves the production, and diffuse distribution of ‘plasticity-proteins’, which are captured and utilised only at those synapses possessing a tag. This hypothesis was tested by stimulating two independent, but overlapping inputs to CA1 pyramidal cells in hippocampal slices. L-LTP was induced on one pathway (S1), and then anisomycin was added to the bath just before the second pathway (S2) was tetanised. Normally, in the presence of anisomycin, L-LTP would be inhibited, but these experiments revealed that not only was L-LTP induced on S1, S2 also remained potentiated for up to 8 hr post-tetanus.

Subsequent experiments demonstrated that ‘weak’ stimulation, which normally results in E-LTP could be heterosynaptically ‘transformed’ into L-LTP if a ‘strong’ tetanus was delivered to an independent input to the same population of CA1 pyramidal cells (Frey and Morris, 1998a). The ability of heterosynaptic stimulation to transform a normally short-lasting potentiation into L-LTP was also found to be dependent upon the interval between ‘weak’ and ‘strong’ stimulation. When ‘strong’ stimulation was delivered within 1 - 2 hr of the ‘weak’ stimulus, L-LTP was induced on both pathways, however this effect declined at longer intervals. Further discussion of these experiments follows in Chapter 5.

The ‘synaptic tag’ hypothesis differs from both the ‘mail’ and ‘local synthesis’ hypotheses in a number of important respects. The suggestion made by the ‘mail’ hypothesis that the persistence of LTP is determined specifically at the time of induction is particularly relevant to the experiments that are reported in this thesis (see Chapters’ 4, 5 and 6). Although protein synthesis may be upregulated at the time of the LTP inducing tetanus, this may be more of an experimentally convenient method of investigating the persistence of LTP rather than reflecting the situation *in vivo* where the levels of proteins may vary dynamically over a wider time scale. From this alternative viewpoint, the variable persistence of LTP results from the interaction of two events. The first involves the setting of synaptic tags that are only active for a limited period of time. Secondly, the time taken for the distribution of ‘plasticity-proteins’ to dendrites and the resulting local interactions that stabilises the potentiated state of the

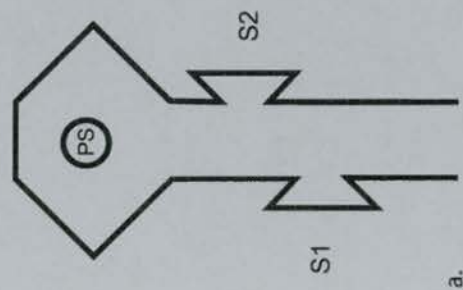
synapse. This allows for a more dynamic modulation of the persistence of LTP, and the possible regulation of these events by neuromodulatory inputs to the hippocampus.

The 'local synthesis' hypothesis is not entirely inconsistent with the 'synaptic tag' hypothesis in one respect. Locally synthesised proteins may be captured by the synaptic tag thereby reducing the distance between the synapse and the area where new proteins are being produced. However, a tag may not even be necessary if all that is required is that the locally residing mRNA deliver proteins to proximal dendritic spines. However, this cannot explain one critical finding of the synaptic tag experiments. Namely, when protein synthesis was inhibited at the time of LTP induction on one pathway (S2), prior stimulation of an independent pathway (S1) resulted in L-LTP being induced on both S1 and S2 (Frey and Morris, 1997). Both local and somatic synthesis would have been shut down at the time of the S2 tetanus; therefore local dendritic translation could not explain the persistence of L-LTP on this pathway (S2). Therefore, the tag is unlikely to be a newly synthesised protein.

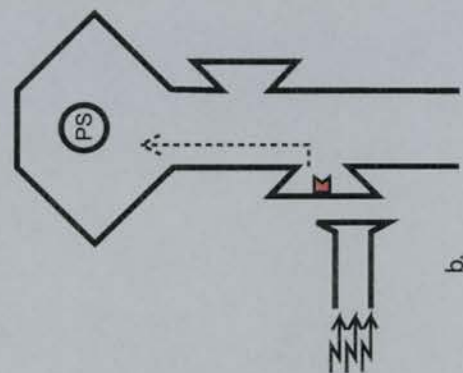
Figure 1.3 An alternative solution to the problem: The synaptic tag hypothesis.

The 'synaptic tag' hypothesis proposes that the persistence of LTP at any group of synapses (a) is a function of two dissociable events. Experiments demonstrated that high frequency stimulation of one input S1 (b) resulted in L-LTP at these synapses via the setting of a synaptic tag and the distribution of 'plasticity-proteins' throughout the cell. Subsequent high frequency stimulation of an independent pathway, S2, also resulted in the tagging of these synapses, but this time protein synthesis was blocked by anisomycin (c). However, the tags set at S2 are able to capture the proteins that had been previously been distributed in response to the S1 tetanus (d), and so L-LTP is induced in S2 also.

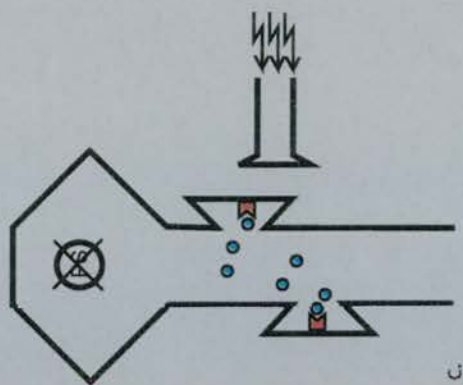
Subsequent experiments (e) demonstrated that 'weak' activation of one pathway, S1, resulted in the tagging of these synapses (f) such that when 'strong' stimulation was delivered to an independent pathway, S2, the proteins triggered by this high frequency tetanus (g) are captured by both the S1 and S2 tagged synapses.



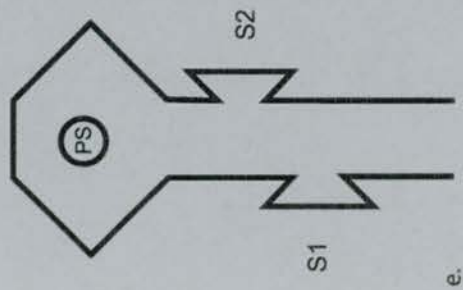
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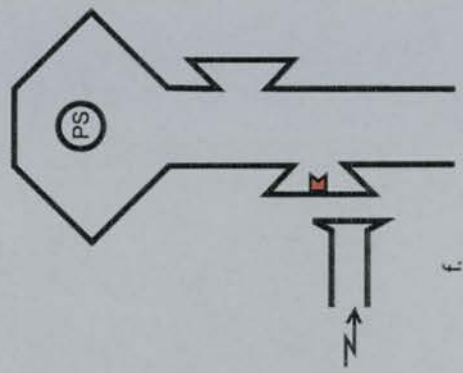
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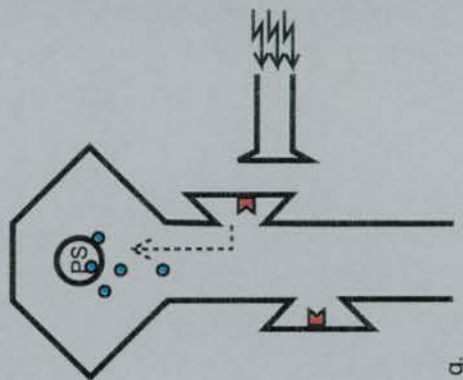
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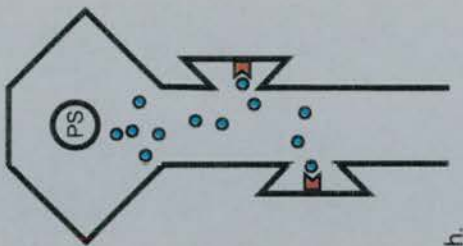
e.



f.



g.



h.

1.12.4 The identity of the 'synaptic tag'.

The validity of the synaptic tag hypothesis was confirmed in subsequent experiments with hippocampal slices (Frey and Morris, 1998a), and evidence in favour of such a mechanism has also been found in *Aplysia* (Martin et al., 1997) where a single puff of serotonin to one branch of a single bifurcated cultured neuron results in short-lasting facilitation. If repeated puffs of serotonin are applied to another branch, the facilitation produced by a single puff is now long-lasting. The findings of Frey and Morris, (1998a) suggest that the tag(s) decay over 1-2 hr; however, the precise identity of the tag(s) is not known.

Nonetheless, there are a number of criteria which potential tags must fulfil. First, the activated tag must be spatially restricted to only those synapses active during the induction of LTP. Second, the tags should decay with time, and as a consequence, be reversible. Third, the tag must be able to interact with the 'plasticity-proteins' which are critical to maintaining the potentiated state of the synapse for periods longer than those associated with the setting of tags. Fourth, these interactions must be the basis of the stabilisation. With these considerations in mind, a number of possibilities exist.

Synaptic tags may take the form of persistently active kinases, which remain activated even after the signal that initially generated their activation. The requirement would be that in addition to the molecule's effects on synaptic transmission, it would also function to capture proteins. Many of the protein kinases that have already been discussed in earlier sections show persistent activation after the triggering signal. The persistent activation of these kinases induced by synaptic activity could mark synapses and enable proteins to selectively target only those synapses where such persistent activity is occurring, or has recently occurred. PKA may fit this role given the evidence for a 'gating' function of this molecule (Blitzer et al., 1995). The PKA pathway has been shown to be involved in the maintenance of L-LTP (Frey et al., 1993; Huang and Kandel, 1994; Nguyen and Kandel, 1997), and in *Aplysia*; the ability of a single puff of serotonin to capture proteins synthesised in response to 5 puffs depends on PKA (Casadio et al., 1999). As was stressed in the earlier sections, these different second messenger pathways may overlap and act in concert in order to trigger the downstream effects responsible for the maintenance of LTP (evidence that the MAPK pathway is a point of convergence for these different signalling pathways was discussed before Impey et al., 1999; Roberson et al., 1999; Giovannini et al., 2001). In addition to marking activated synapses, a signal must be sent to the nucleus to activate transcription and translation. In some cell types,

the catalytic subunits of PKA have been shown to translocate to the nucleus from the cytoplasm (Nigg et al., 1985; Hagiwara et al., 1993). Using confocal imaging techniques real time images of cAMP and PKA migrations within *Aplysia* neurons have also been observed (Bacskai et al., 1993). PKA labelled with the fluorescent dyes fluorescein and rhodamine on the catalytic (C) and regulatory (R) subunits respectively, were injected into *Aplysia* sensory neurons. The ratio of fluorescein to rhodamine emissions (imaged with a confocal microscope) provides an indication of the extent of the dissociation of the PKA, R and C subunits and thus a measure of the cAMP levels (which regulate this dissociation) within the cell. In response to application of 5-HT, cAMP levels increased, resulting in a translocation of the PKA catalytic subunits into the nucleus. Earlier sections also described how calmodulin may also translocate to the nucleus to activate the transcription factor CREB (Deisseroth et al., 1996; Deisseroth et al., 1998). Little is understood if, and how any of these activated kinases might interact with the diffusely distributed proteins.

Another candidate is a morphological change in the dendritic spines that were activated during the induction of LTP. Various different morphological alterations have been associated with LTP (for reviews see Bailey and Kandel, 1993; Yuste and Bonhoeffer, 2001; Marrone and Petit, 2002). Changes in dendritic spines that are activated in response to LTP inducing stimuli may mark specific synapses in a way that the shape of these spines allows for easier access of the ‘plasticity-proteins’ that are necessary for the long term alterations in synaptic strength. Many studies have reported no overall changes in the number of synapses per neuron following LTP induction (Desmond and Levy, 1983, 1986a, b; Weeks et al., 1999, 2000, 2001). Although some form of synaptic turnover is associated with synaptic plasticity, this appears to be balanced by a proportionate process of synaptic pruning. What appear to be of greater functional importance are changes in the proportion of different types of synapses in terms of their geometry. Toni et al., (1999) found that LTP induction resulted in a sequence of morphological changes with a transient increase in synapses with perforated postsynaptic densities (PSDs) followed by multiple spine boutons (MSBs) in which two spines arise from the same dendrite. Weeks et al., (1999, 2000, 2001), found that stimulated synapses become perforated for only a short duration (1 hr) following LTP induction. Geinisman et al., (1991, 1993) found an increase in the number of perforated synapses per neuron 1 hr following LTP induction in the dentate gyrus *in vivo*. This increase at 1 hr was not evident at 13 days post-tetanus (Geinisman et al., 1996). These morphological changes may arise due to changes in the actin cytoskeleton or adhesion molecules, which are important for remodelling the synaptic architecture. Changes in the actin microfilament network are likely to underlie the

growth of new synaptic structures that has been observed after repetitive stimulation of hippocampal synapses (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999).

One suggestion that has been made is that the synapses with a perforated PSD could represent a morphological correlate of the mechanisms of receptor trafficking proposed to contribute to LTP (Luscher et al., 2000). In support of this, Toni et al., (2001) found that LTP in cultured hippocampal neurons is associated with a transient increase in the proportion of perforated synapses that was evident 30 min after the induction of LTP. They also observed that a high proportion of these perforated synapses contain coated vesicles which they suggest could represent a correlate of the mechanism of recycling leading to the incorporation of new receptors into the synaptic membrane. It is interesting therefore that Nayak et al., (1998) reported that L-LTP was associated with an increase in synthesis of AMPA receptor subunits. Synthesis of AMPA receptor subunits was measured by assaying [³⁵S]-methionine incorporation into the GluR1, and GluR2/3 subunits at different times after the induction of L-LTP. The synthesis of both GluR1 and GluR2/3 was significantly higher than unstimulated controls after 3 hr, and both PKA and transcriptional inhibitors blocked this increased expression. As mentioned above, an essential requirement of candidate synaptic tags is that they decay with time and are reversible. The evidence reported above where changes were seen after 1 hr, and had disappeared after 13 days (Geinisman et al., 1991; Geinisman et al., 1993; Geinisman et al., 1996), would fit this, and very transient changes have also been reported by others (Weeks et al., 1999, 2000, 2001). However, it is yet to be understood if gene expression or other cell-wide events are involved in stabilising these local changes.

A relatively recent idea has emerged from work on the crayfish neuromuscular junction, where a synaptic tag that allows a neuron to integrate synaptic stimulation at a single synaptic connection over time has been described. Beaumont et al., (2002) found that presynaptic I_h potassium channels become activated during tetanic stimulation and this generates a synaptic tag, which allows the motor neuron to increase transmitter output by subsequently responding to further stimuli that would otherwise be ineffective. They also found that if the activation of the I_h channels occurs concomitantly with elevations in presynaptic calcium, the tagged synapses can undergo protein synthesis dependent long-term facilitation in response to subthreshold stimuli that are applied at least 1 hr after the tag was set. Therefore, not only does the tag provide a history of the activity at the synapse it allows for the combination with subthreshold stimuli, within a certain time-window, to produce a long-lasting change in

synaptic efficacy. This long-lasting change at the neuromuscular junction is dependent on local protein synthesis and not transcription (Beaumont et al., 2001). As suggested earlier, local dendritic protein synthesis may require the presence of a synaptic tag in order to generate long-lasting changes in synaptic strength.

The other likely candidate synaptic tag involves the local regulation of protein synthesis within dendrites. There are two possibilities; firstly, mRNAs synthesised in the cell body are delivered to dendrites that are said to possess all the translational machinery, thereby enabling the persistence of LTP to be locally regulated. A tag may be involved in regulating the translation of proteins from these mRNAs. A second possibility is that the mRNA is synthesised locally within the dendrite and therefore the persistence of L-LTP may be in some situations, independent of somatic protein synthesis. Local translation could potentially meet the spatial and temporal requirements of the candidate tag(s), and it is possible that the interaction with somatically synthesised mRNAs arises as these molecules target only those synapses where translation is underway. Although the evidence from the experiments by Frey and Morris, (1997) is inconsistent with the idea that the tag is itself a locally translated protein, there is some controversy surrounding this. Recent work by Sherff and Carew, (1999) suggests that a stimulus that is subthreshold for the induction of lasting long-term facilitation in *Aplysia*, is associated with local protein synthesis and that these proteins contribute to the generation of long-term facilitation, i.e., a protein synthesis dependent tag. Evidence for this comes from experiments where a pulse of serotonin to the synapse only results in short-term facilitation, and similarly, when applied to the soma, serotonin did not produce long-term facilitation. However, when the serotonin pulse to the synapse overlapped with the somatic pulse, significant long-term facilitation was observed. This requirement for overlap between the somatic and synapse activation required protein synthesis at the synapse but not the cell body during the serotonin pulse to the synapse. The field of dendritic protein synthesis is a growing focus for much current research and so is considered in greater detail.

1.13 Local protein synthesis.

There is growing interest in the idea that dendritic protein synthesis may underlie the persistence of L-LTP independently of somatic distribution and translation of proteins. The initial interest in the possibility for local regulation of protein synthesis was initiated by the discovery of polyribosomes and the associated machinery for translational regulation beneath

postsynaptic sites on dendrites (Steward and Levy, 1982; Steward, 1983; Steward and Fass, 1983). Experiments using quantitative electron-microscopic analyses have shown that most of the ribosomes located within dendrites are in an optimal position to be influenced, and be influenced by synaptic activity (Steward and Levy, 1982). They are located at the base of dendritic spines, from which the spine neck protrudes. Many of these ribosomes have been shown to be associated with organelles similar to the rough endoplasmic reticulum in the cell body, which is an important component of the translational machinery. These polyribosomes are abundant in the dendrites of developing neurons which might suggest that they contribute to synapse formation in these immature neurons, although this is not certain (Steward and Falk, 1986). A very recent study by Ostroff et al., (2002) has demonstrated that in hippocampal slices from young animals, the percentage of dendritic spines containing polyribosomes increased after tetanic stimulation.

1.13.1 Dendritically localised mRNAs.

Experiments using in situ hybridisation have been used to determine the presence of particular mRNAs in dendrites. Although the presence of a particular mRNA in dendrites does not confirm that the mRNA is translated at synapses. A number of different mRNAs have been identified by these methods. There is a diverse range of mRNAs that encode unrelated proteins including cytoplasmic, cytoskeletal, integral-membrane, and membrane-associated proteins that may be involved in a variety of processes other than the mechanisms of synaptic plasticity. These different mRNAs may also be developmentally regulated, for instance, the mRNA for calmodulin can be detected by in situ hybridisation in dendritic laminae of developing, but not mature animals (Berry and Brown, 1996). The first mRNAs to be identified as being localised within dendrites were the mRNAs that encode microtubule-associated protein 2 (MAP2), (Garner et al., 1988) and the mRNA for the α -subunit of CaMKII (Burgin et al., 1990). Many other mRNAs have since been documented to show extensive dendritic localisation in hippocampal neurons (for a review, see Steward and Schuman, 2001).

In situ hybridisation techniques for detecting mRNA within dendrites may not be sufficient to determine the complete complement of mRNAs within dendrites and only two cRNA probes can be applied to the tissue at any time. Also, some mRNA species may be expressed in low levels and may be undetectable by in situ techniques. Miyashiro et al., (1994) developed a technique whereby patch pipettes are used to aspirate the cytoplasmic contents of individual

neurons in culture. RNA amplification is then used to clone the mRNAs present. A large, heterogeneous population of mRNAs have been detected using this method, (Crino and Eberwine, 1996) many of which remain to be characterised and were not detected by in situ hybridisation. Whether this is because they are present at low levels not detected by the hybrid probes, or due to the fact that Miyashiro et al., (1994) analysed the cytoplasm of immature cultured neurons, which may possess a different complement of mRNAs to the mature neuron is not clear. An intriguing finding reported by Crino et al., (1998) involving cultured hippocampal neurons, revealed that mRNAs encoding the transcription factor CREB were identified in distal dendrites. Transcription factors are traditionally considered to be specifically active in the nucleus and cell body. Although, transcriptional regulation may therefore be possible within dendrites, the authors suggest that the transcription factor proteins corresponding to the dendritically localized mRNAs are synthesised, post-translationally modified, and then transported to the nucleus where they modulate subsequent gene transcription. The presence of endogenous CREB protein in dendrites has not been reported so far.

1.13.2 How are mRNAs transported to dendrites?

The strongest evidence for dendritic mRNA transport has come from studies of the immediate early gene *Arc*. The projection from the entorhinal cortex to the dentate gyrus is highly topographically organised. The medial entorhinal cortical projections terminate specifically in the middle molecular layer of the dentate, and the lateral entorhinal projection terminating in the outer molecular layer. When LTP was induced in the medial perforant pathway *Arc* expression was induced, and the newly synthesised mRNA specifically migrated into dendrites in the middle molecular layer. Activation of afferents that terminate in different regions, induced *Arc* localisation only in these corresponding regions (Steward et al., 1998). Local accumulation of *Arc* protein was also demonstrated, however it is not known whether the protein is targeted to the postsynaptic sites in the active region.

The targeting of specific mRNAs into dendrites often requires *cis*-elements in the mRNA. For example, the 3' untranslated region (UTR) of α CaMKII mRNA is required for the dendritic localisation of this mRNA (Mayford et al., 1996a). A very recent study where the 3' UTR of the α CaMKII was mutated without affecting the protein coding region showed that the ability of the mRNA to localise specifically within dendrites was disrupted (Miller et al., 2002). In these animals the mRNA for α CaMKII is restricted to the soma and was associated with a

reduction in α CaMKII in the postsynaptic density and although L-LTP could be induced, the magnitude of the potentiation was reduced. Several proteins that are involved in mRNA targeting have been described in *Drosophila* and *Xenopus*. One such molecule, *stau*, has been found to be concentrated in the dendrites of cultured hippocampal neurons where it is associated with endoplasmic-reticulum like structures in the postsynaptic density (Kiebler et al., 1999). *Staufen* protein particles that contain mRNA have been visualised to move along microtubules in dendrites (Kohrmann et al., 1999), which may enable dendritic mRNA targeting. Green fluorescent protein was tagged to *stau* (*Stau*-GFP) and transfected into hippocampal neurons. The *Stau*-GFP formed aggregates that co-localised with the fluorescent dye SYTO14. Some of the granules were seen to move in a bi-directional manner between the cell body and dendrites of the cultured neurons.

1.13.3 mRNA translation in dendrites.

Following the transport and localisation of mRNAs in dendrites it is important to determine whether or not translation of the associated protein occurs. In a culture system that allowed for the separation of living axons and dendrites from their cell bodies, Torre and Steward, (1992) pulse labeled isolated dendrites with ^3H -leucine for 30 min and then fixed the dendrites to identify sites of protein synthesis. Autoradiographic analyses revealed that isolated dendrites (immunochemically identified using antibodies against MAP2) became heavily labeled, whereas axons exhibited little if any labeling. The labeling was essentially eliminated when the neurites were pulse labeled with ^3H -leucine in the presence of protein synthesis inhibitors.

There is considerable evidence that α CaMKII mRNA is locally translated in dendrites. Ouyang et al., (1997, 1999) observed increased immunostaining for both phosphorylated and non-phosphorylated CaMKII in dendrites following tetanic stimulation of hippocampal slices. The increase in non-phosphorylated CaMKII occurred within 5 min of the tetanus and was blocked by anisomycin, therefore suggesting that the kinase was dendritically synthesised. In a related study, Steward and Halpain, (1999) revealed that tetanic stimulation of the perforant path *in vivo*, caused increases in staining for α CaMKII in the activated dendritic laminae. This increase in the staining of α CaMKII was not inhibited by anisomycin; therefore the locus of synthesis is not clear. The possibility that newly synthesised proteins were actually produced in the cell body and rapidly transported into the dendrites cannot be ruled out.

1.13.4 Synaptic plasticity and dendritic protein synthesis.

One of the most convincing demonstrations linking dendritic protein synthesis to synaptic plasticity involved the potentiation produced by the growth factors brain derived neurotrophic factor (BDNF) and neurotrophin-3. Hippocampal slices from transgenic mice that lack BDNF do not display L-LTP (Korte et al., 1998). Kang and Schuman, (1995) found that application of either BDNF or neurotrophin-3 to CA1 synapses caused a long-lasting (2-3 hr) enhancement of synaptic transmission. Pre-treatment with a protein synthesis inhibitor blocked both the early (within 10 min) and late phases (2-3 hr) of this form of potentiation (Kang and Schuman, 1996). Microlesions were then performed to separate the synaptic neuropil from the cell bodies, and these isolated preparations still exhibited growth factor induced potentiation that was sensitive to protein synthesis inhibitors suggesting that the site of synthesis is locally within dendrites and not the cell body.

Greater temporal resolution in observing protein synthesis in dendrites has been provided by a recent study by Aakalu et al., (2001). They used green fluorescent protein (GFP) that was flanked by the 3' and 5' UTR of the α CaMKII subunit, in combination with time lapsed microscopy to investigate local protein synthesis in cultured hippocampal neurons. In order to rule out the cell body as a potential source of the GFP signal they used two methods for isolating the dendrites from the cell bodies. The first involved surgical transections, and in the second, GFP in the cell body was continuously photobleached, while fluorescence was monitored in the distal dendrites. BDNF application in both cases resulted in increased GFP expression in the isolated dendrites.

1.14 Recent developments and directions in synaptic tagging and L-LTP.

The synaptic tag hypothesis has generated a lot of interest in the field of synaptic plasticity and memory. A number of recent experiments by different groups specifically relate to the ideas central to the mechanisms of long-term synaptic modifications.

1.14.1 The 'plasticity-proteins' sequestered by the tag.

Earlier, evidence was presented focussing on the role that the transcription factor CREB plays in both long-lasting synaptic plasticity and long-term memory. Further insights into the possible interaction of transcriptionally induced gene products with synaptic tags have been

suggested in a recent study by Barco et al., (2002). This work involved transgenic mice with a forebrain restricted and temporally regulated expression of a constitutively active form of CREB called VP16-CREB. VP16-CREB was expressed selectively in CA1 hippocampal neurons such that, only these cells showed a reduced threshold for inducing L-LTP, and the induction of L-LTP was independent of transcription. A weak tetanus capable of inducing only decremental E-LTP in normal mice induced L-LTP in VP16-CREB animals. The authors suggest that CREB activation turns on downstream genes, and the products of these genes are captured by the activated, tagged synapses, resulting in L-LTP. Future experiments to determine if expression of VP16-CREB effects long-term memory in a manner consistent with its effects on LTP would be worthwhile.

1.14.2 Back-propagating action potentials and L-LTP.

A recent experiment by Dudek and Fields, (2002) re-addresses the ‘synapse to nucleus’ question. Earlier, evidence was reviewed based on the idea that in order to achieve long-lasting forms of synaptic plasticity, a signal generated at the synapse is required to be sent to the nucleus in order to initiate the cascade of events leading to transcription and translation. Dudek and Fields, (2002) propose an alternative, “*An alternative possibility is that action potential firing in the postsynaptic neuron might suffice to activate gene expression by influx of calcium through voltage-sensitive calcium channels in the cell membrane*” (p. 3962). This statement is contrary to the findings reported earlier from experiments by Deisseroth et al., (1996) who reported that phosphorylation of CREB appears to be dependent upon synaptic stimulation and not action potential firing. When Ca^{2+} transients were monitored with fluo-3 Ca^{2+} imaging, increases in Ca^{2+} were seen in the cytoplasm and nucleus in response to high frequency stimulation even when AP5 and CNQX were present, i.e., a condition in which CREB phosphorylation was blocked. Increases in nuclear Ca^{2+} do not seem to be sufficient to trigger CREB phosphorylation. Using antidromic stimulation to back-fire the CA1 cells in the alveus, Dudek and Fields, (2002) revealed that this stimulation in itself did not induce LTP, it did however rescue a normally decremental LTP when the E-LTP inducing synaptic stimulus was delivered 1 – 2 hr after the antidromic stimulation to back-fire the cells.

1.14.3 Metabotropic glutamate receptors and synaptic tagging.

Experiments by Behnisch et al., (1991) found that the maintenance of L-LTP was affected by application of the metabotropic glutamate receptor (mGluR) antagonist D,L-AP3 to

hippocampal slices. Allied to this is the finding that “priming” activation of group 1 mGluRs will transform a decremental form of LTP into a more persistent form (Bortolotto et al., 1994; Cohen and Abraham, 1996; Bortolotto and Collingridge, 1998; Cohen et al., 1998). Recent experiments by Raymond et al., (2000) have taken this further. Application of the selective group 1 mGluR agonist DHPG to hippocampal slices primed the subsequent induction of LTP with a weak tetanus, such that the potentiation induced after the drug treatment was more persistent compared to untreated controls. This priming of LTP was blocked when the translational inhibitor emetine was applied before, and during the DHPG application. The subsequent induction of LTP was unaffected but LTP was decremental after concurrent treatment with emetine. This suggests that DHPG triggers protein synthesis before the induction of LTP that then interact with the events triggered by the tetanus leading to non-decremental LTP for 2 hr. One possibility is that the proteins synthesised by the activation of group 1 mGluRs are subsequently captured at the synapses tagged by the induction of LTP leading to non-decremental LTP being induced.

The next set of experiments asked whether synaptically released glutamate activates mGluRs in a way that the subsequent induction of LTP is also primed (Raymond et al., 2000). Slices were primed by the delivery of a theta burst stimulus in the presence of the NMDA receptor antagonist AP5. Synaptic priming in this manner enhanced subsequent LTP compared with AP5 treated controls given the same weak tetanus. The synaptic priming effect was blocked when either the group 1 mGluR antagonist AIDA, or emetine were applied before and during the priming stimulation. The authors suggest that local dendritic protein synthesis is the mechanism by which the priming stimuli facilitate the subsequent induction of LTP with weak stimuli. As the original synaptic tag experiments of Frey and Morris, (1997, 1998a) reveal, the persistence of LTP may be heterosynaptically modulated by the delivery of proteins to synapses activated before or after the trigger leading to translation. (Raymond et al., 2000) addressed this by delivering theta burst stimuli to one pathway in the presence of AP5; after washout of the AP5, LTP was induced with a weak stimulus to a second independent pathway. Although the initial induction of LTP on this independent pathway was facilitated compared to controls, its persistence was not. They suggest that the proteins synthesised in response to the priming stimulus are locally synthesised at the synapses activated by the priming stimulus. mGluR dependent LTD has been reported to be dependent on rapid dendritic protein synthesis also (Huber et al., 2000).

1.15 The present experiments.

The focus of the present experiments was on understanding the heterosynaptic modulation of L-LTP by demonstrating that L-LTP could be induced when one might normally expect to observe decremental E-LTP.

In order to address some predictions of the synaptic tag hypothesis, it was first essential to develop a system for maintaining hippocampal slices *in vitro* for > 8 hr. Once this was reliably achieved, experiments related to the synaptic tag hypothesis were undertaken. The first aim of experiments was to demonstrate input specific long-lasting potentiation following multiple high frequency stimulation to one pathway, and the stability of a second non-tetanised control pathway for a minimum of 6 hr post-tetanus. Once this L-LTP could be induced with reasonable reliability, it was important to determine the induction parameters sufficient to induce a shorter lasting decremental LTP, or E-LTP.

Having established these first two important steps, experiments were focussed on exploring the nature of synaptic tagging in more detail. Firstly, is it possible to demonstrate synaptic tagging in hippocampal slices by inducing decremental E-LTP in one input pathway, and demonstrate that this potentiation can be heterosynaptically influenced by either prior or subsequent high frequency stimulation of an independent pathway? Is the ability of heterosynaptic stimulation to influence the potentiation of an independent pathway is due to the activation of protein synthesis by this heterosynaptic stimulus? Also, do these protein synthesis inhibitors block the incorporation of radiolabelled amino acid (i.e., [³⁵S]-methionine) into hippocampal slices?

Evidence was discussed focusing on the way in which LTP may be modulated by a variety of different inputs to the hippocampal formation. One important pathway is the dopaminergic projection to the hippocampus. Dopamine may be a key modulatory transmitter underlying the heterosynaptic mechanisms involved in synaptic tagging. Experiments were aimed at characterising the role of dopamine in synaptic tagging and L-LTP. If dopamine is released by high frequency stimulation it may be possible to demonstrate synaptic tagging even when the NMDA receptor has been blocked. Stimulation in the presence of AP5 may block the induction of L-LTP on this pathway but may enable the subsequent stimulation of an independent pathway to induce L-LTP even though this would not ‘normally’ occur. One way

in which dopamine may be important in modulating L-LTP is by stimulating protein synthesis. The final series of experiments involved testing whether synaptic tagging and the paradoxical induction of L-LTP occurs even when the dopamine D1 receptor antagonist SCH23390 is present. It was therefore necessary to demonstrate that L-LTP could be blocked by application of SCH23390 to hippocampal slices. A key new set of experiments involved testing whether it is possible to 'paradoxically' induce dopamine receptor-dependent L-LTP even during the presence of SCH23390 which 'normally' blocks L-LTP in a similar design as the experiments of Frey and Morris, (1997).

Long-term, but not short-term memory is dependent upon protein synthesis. A similar requirement has been described for L-LTP but not E-LTP. As dopamine appears to be important for L-LTP it may also play a role in long-term memory. One possibility is that the dopaminergic projection from the ventral tegmental area to the hippocampus may regulate the persistence of memories that are hippocampal dependent. Experiments were conducted to test the effects of intra-hippocampal infusions of SCH23390 on a novel version of the watermaze i.e., the delayed matching-to-place task. These experiments were the first step towards developing a behavioural analogue of synaptic tagging, and they provided the basis for future experiments to investigate the possible behavioural implications of synaptic tagging.

Chapter 2. Materials & Methods.

2.1 Electrophysiology.

2.1.1 The *in vitro* hippocampal slice.

The mammalian brain slice preparation has become a standard tool for investigating the physiology and pharmacology of neurons, and the properties of neuronal circuits. Recording from brain slices was first developed by (Yamamoto and McIlwain, 1966) who showed that isolated slices of prepyriform cortex could be maintained *in vitro* and still show electrical activity comparable to that seen in the whole animal. (Yamamoto and Kawai, 1967) and Bliss & Richards (1971) first applied the technique to the hippocampus. Skrede & Westgaard (1971) refined the application of the technique to the hippocampus; it has since become the number one candidate for brain slice studies.

There are many reasons for choosing the *in vitro* hippocampal slice rather than intact animals for certain experimental procedures. Among these advantages are: (a) The environment (i.e. the incubating media, temperature, pH, oxygenation) in which the slices are maintained can be specified and/or changed in a carefully controlled manner; (b) Even low powered microscopes allow the investigator good visual resolution of specific anatomical structures, including presynaptic fibre tracts and cell body layers. This allows for controlled positioning of both stimulating and recording electrodes within the area of investigation; (c) Many slices from a single hippocampus can be prepared and studied in one experiment. All will have the same genetic and experimental history prior to preparation; (d) There are no mechanical disturbances, such as those caused by heartbeat and respiration, to hinder recording; (e) Drugs can be applied to brain slices in a highly controlled manner. The concentration and duration of application can be controlled with great accuracy. In the intact animal, the varying metabolic processing of drugs reduces such control. Interactions with other structures rather than just the one of interest may also confound experimental results; (f) No anaesthetics or foreign agents need be applied to the slice during recordings, as is the case *in vivo*, thereby minimising the potential interaction of these agents with processes in the intact animal, or interactions with drugs of interest.

The aim of the preparation and incubation of hippocampal slices within various recording chambers is to maintain the tissue in a state that is reasonably close to the situation *in vivo*. Factors that may influence slice health include (i) the age of the animal; (ii) the specific composition of the incubation media; (iii) whether slices are maintained at the interface of the media and oxygen or submerged; (iv) the method of anaesthesia, killing and cutting the slices (see (Garthwaite, 1980; Reid et al., 1988; Lipton, 1995)).

There is a substantial amount of damaged tissue in hippocampal slices (Frotscher et al., 1981). Neurons appear swollen in the interior portion (Ibata et al., 1971), and this tendency increases as slice thickness increases from 300 to 700µm (Bak et al., 1980). The majority of the morphological analyses of slices have been carried out on slices incubated for approximately 2 hr. The experiments reported in this thesis extend well beyond these time periods and with the relatively long pre-incubation periods as used here (i.e., 2.5 hr), these changes may stabilise once the experiment is begun. Many laboratories conducting L-LTP experiments use the 1.5 – 2 hr pre-incubation periods e.g. (Nguyen et al., 1994; Impey et al., 1996; Abel et al., 1997; Winder et al., 1998; Rosenblum et al., 2002). Kirov et al., (1999) confirmed that no physiological responses can be elicited from slices during the first 10-15min after transfer to a recording chamber, regardless of stimulus intensity. With recovery during the next 30 min, fEPSPs stabilised over the next 1 hr in slices from young animals. An interesting finding from these experiments is that, when slices are prepared and incubated in the standard way for *in vitro* recordings, new synapses are formed. However, the number of new synapses was found to be maximally elevated by ~2 hr *in vitro* and remained stable for up to 13 hr.

2.1.2 Animals and preparation of slices for *in vitro* electrophysiology.

For all *in vitro* electrophysiological experiments reported in this thesis, male Wistar rats (age 7-8 weeks) were used for the preparation of transverse hippocampal slices. Animals were housed in groups of 3-4 per cage, and were maintained on a 12:12h light: dark cycle, with lights on at 08:00 h. Food and water was supplied ad libitum. A different strain, the Lister Hooded rat, was used for behavioural experiments. This strain has a pigmented retina and much better vision required for behavioural training. There is no reason to suspect any substantial differences in hippocampal function or mechanisms across these two different strains used in the experiments reported here.

For preparation of slices, animals were sacrificed with Schedule 1 procedures in accordance with the Home Office animals (scientific procedures) act 1986. The brain was quickly removed (~1 min) and placed in a Petri dish containing cold (0-4°C), oxygenated (95% O₂ / 5% CO₂), artificial cerebrospinal fluid (aCSF) of the following composition: NaCl 120mM, KCl 3mM, MgSO₄ 2mM, CaCl 2mM, NaH₂PO₄ 1.2mM, NaHCO₃ 23mM, D-Glucose 11mM. The stock solution from which the aCSF was prepared each day was a ×10 solution, stored in the fridge, which did not contain either NaHCO₃ or D-Glucose, both were added fresh each day of an experiment. Stock solutions were freshly made every 3 weeks. In order to keep the temperature of the aCSF in the Petri dish cold throughout the dissection procedure it was placed in an aluminium cooling block that had been kept in the freezer at -20°C.

In order to keep the tissue cold; the removal of the hippocampus from the rest of the brain was performed submerged in the Petri dish containing the aCSF. The brainstem and cerebellum were removed and the brain placed on its ventral surface. A transverse cut was made to free the forebrain. Fine forceps were inserted into the left hemisphere of the brain to give mechanical stability. An incision was then made in the sagittal plane, along the midline, to separate the two hemispheres. Care was taken to avoid separating both hippocampi at this point. Using dental instruments, the cortex over the right hippocampus was gently pushed away to expose the underlying hippocampus. A further sagittal cut was made to separate the two hippocampi and the right hippocampus was gently freed from the remaining cortical tissue.

The right hippocampus was removed from the aCSF with a spatula and placed on a piece of moistened filter paper that was placed on the chopping block of a Stoelting tissue chopper. The position of the tissue in relation to the blade was adjusted so that the longitudinal axis of the hippocampus was oriented at 30-40° to the blade. The blade was moistened with some aCSF by running a small artist's sable paintbrush (number 1) over the blade. Slices were cut by simply dropping the blade of the chopper onto the surface of the tissue. Approximately 5-6 transverse slices 400µm thick were cut from the middle section of each hippocampus. Each separate slice was gently removed from the blade with the paint brush by a gentle curved rolling motion to lift the slice from the surface with minimal disturbance, and to avoid impaling the slice on any of the hairs of the brush. They were then immediately transferred to a dish containing cold, oxygenated aCSF (0-4°C). Two slices were then transferred, again using the artists brush, to an interface

recording chamber (Scientific Systems Design Inc.), where they were maintained for 2.5 – 3 hr at 32°. The time taken from the point when the animal was killed to the point where the slice was positioned in the chamber for incubation, was approximately 5-6min.

2.1.3 The recording chamber.

There are some general requirements that any *in vitro* slice chamber must fulfil in order to maintain physiological slices. In general, viable hippocampal slices are obtained if the recording chamber provides a moist, oxygenated environment, at a constant temperature throughout the duration of the experiment, and a constant, regular perfusion of the slices with aCSF. Mechanical stability, so to avoid any movement of the slice is also important. These factors are generally within experimental control due to developments in the technology of recording chambers. However, controlling variables such as air bubbles under the slices, which are catastrophic for stable recordings, are sometimes beyond experimental control. These factors all become more critical when recordings are to be carried out for > 8 hr as is the case in the experiments reported here. When such bubbles appeared, the stability of recordings was compromised and the experiment discontinued.

There are two main types of recording chamber, each with their own advantages and disadvantages. The first are *interface* chambers. In this design, the slices are maintained at the interface between a layer of slow flowing aCSF and a layer of humidified 95% O₂ / 5% CO₂ gas. The advantages of this type of design include: (1) Due to the slow flow rates, less aCSF is used with smaller dead space, thereby minimising the cost of pharmacological experiments. (2) The surface of the slice is easily visualised under the microscope for electrode placement. (3) The slices rest on either lens tissue or a nylon mesh, thus getting mechanical support and minimal disturbance during recording. There are some disadvantages to the interface design: (1) Due to the low flow rates, rapid changes in the bathing medium (which becomes critical in pharmacological experiments) are problematic. (2) At temperatures well above room temperature i.e. 32°C, drops of condensation may form on the electrodes that are detrimental to the long-term stability of recordings.

Some of the problems associated with interface recordings can be overcome with the other main design of chamber, the *submerged* chamber. In this design, slices are held between two mesh grids, and completely covered by a layer of oxygenated aCSF, which flows at a much higher rate than in the interface design. Advantages of this design include; (1) higher flow rates allow for fast changes in the incubating solution, which is preferable in pharmacological experiments. (2) The temperature of the slice is easier to control, as they are less influenced by changes in the external environment. (3) The problem with condensation on electrodes is minimised as the electrode is covered by the aCSF also. However, a number of important points must be made. (1) The higher flow rate may impose mechanical stress on the slice, added to this is the fact that a mesh holds the slice in position which will disturb both sides of the slice. (2) The surface of the slice is not as easy to see in comparison to slices in interface chambers, thus making positioning of electrodes more problematic. (3) The size of the evoked response will be reduced as a result of shunting of current through the surrounding medium, which also magnifies stimulus artefacts.

An initial aim of this project was to set up an electrophysiological rig for recording for long periods as a way of replicating and advancing work conducted with our collaborators at the Institute for Neurobiology, Magdeburg, Germany (Dr. J.U. Frey). In the Edinburgh lab, no such facilities existed at the beginning of this project, and so a large part of the initial work was concerned with testing various methods and chamber designs, before the stage was reached where stable recordings were possible for > 6 - 8 hr.

2.1.4 The Scientific Systems Design recording chamber.

We are indebted to Dr. Sukhvinder Dhanjal for his continued technical support throughout this project and advice concerning his Scientific Systems Design Chamber. The type of chamber used for the experiments reported here was a modified version of a model supplied by Scientific Systems Design Inc., UK. The original 'un-modified' version was initially tested and various features were discovered to affect the stability of recordings during this phase. After an extended period of problems associated with the long-term stability, and viability, of the slices kept in this chamber, the modified chamber design was adopted. A major factor in this decision arose after consultation with Dr. Karl Bradshaw (National Institute of Medical Research, Mill Hill) who

visited Edinburgh to help set up the 'modified' chamber design. The chamber design is shown in Figure 2.1.

Hippocampal slices ($n = 2$) sit on a small circular piece of lens tissue which is 'moulded' to fit the sloped circular insert. The slices are continuously perfused with oxygenated 95% O_2 / 5% CO_2 aCSF which is warmed as it passes through a water bath at the base of the chamber. The aCSF is pre-warmed to 32°C, as the reservoir in which it is kept is placed in a water bath outside of the rig. The temperature of the aCSF in this water bath is monitored throughout each experiment and remains within a small range i.e., 31.8 - 32.2°C. The purpose of the lens tissue is to give gentle mechanical support to the slices, it also reduces the surface tension of the aCSF allowing even distribution over and around the slices as it flows down the gradient of the sloped insert. To aid steady perfusion and drainage of aCSF from the chamber, a small piece of silver wire was positioned on one side of the exit channel of the sloped insert. This acted as a 'wick' along which the aCSF could flow into the outflow channel. On the opposite side of the insert a similar piece of wire, this time chlorided silver wire, was positioned. This wire acted in a similar manner as previously described, and it functioned as the 'second pole' of the monopolar stimulating electrodes. These measures along with adjustments to a hypodermic needle inserted into the exit channel allowed for control of the fluid level within the recording chamber. The hypodermic needle was connected to tubing leading to a peristaltic pump. Careful adjustments, confirming advice from Karl Bradshaw, showed that a flow rate of approximately 150-200 μ l/min, was optimal to maintain healthy slices for at least 8 hr (and in some cases up to 24 hr, in experiments run overnight). By minimising the absolute volume of aCSF within the chamber, difficulties associated with 'flooding' of the chamber were avoided. The slower flow rate improved the mechanical stability as it minimised 'pulsatile' perfusion of aCSF through the chamber. As the aCSF is oxygenated, the slices obtain oxygen from the solution. However, because aCSF is a poor carrier of dissolved oxygen, the slices also receive oxygen from the 'micro-environment' of the chamber. This is achieved as the gas (95% O_2 / 5% CO_2) is passed through a ceramic bubbler in the base of the chamber. The gas is warmed and moistened as it passes through the water bath in the base and distributed over the slice by vents connecting the base and top section of the chamber. A sloped cover slip directs the gas towards the area of the sloped insert, thereby creating a humidified, oxygenated 'micro-environment' (see Figure 2.2 a +b).

With the chamber that was used in the experiments reported here, the temperature was maintained at 32 °C over the entire course of an experiment. This was achieved by using the heater system built into the chamber, by pre-heating the Krebs solution outside the chamber to 32.3 - 32.5 °C (by placing the reservoir in a water bath), and by insulating the entire rig in aluminum foil. By placing a small electric heater under the air table the temperature within the rig could be maintained at ~30 °C (see Figure 2.2 c). Measurements of the temperature of the recording chamber were taken before placing the slices in the chamber each day, during the experiment, and at the end of the experiment and were confirmed to equal ~ 32.0 – 32.3 °C.

Figure 2.1 The Scientific Systems interface recording chamber.

Pre-warmed (32 °C) aCSF is perfused across the surface of hippocampal slices positioned on the lens tissue insert. In this set-up, the slices are enclosed in a humidified, warm atmosphere containing 95% O₂/5% CO₂.

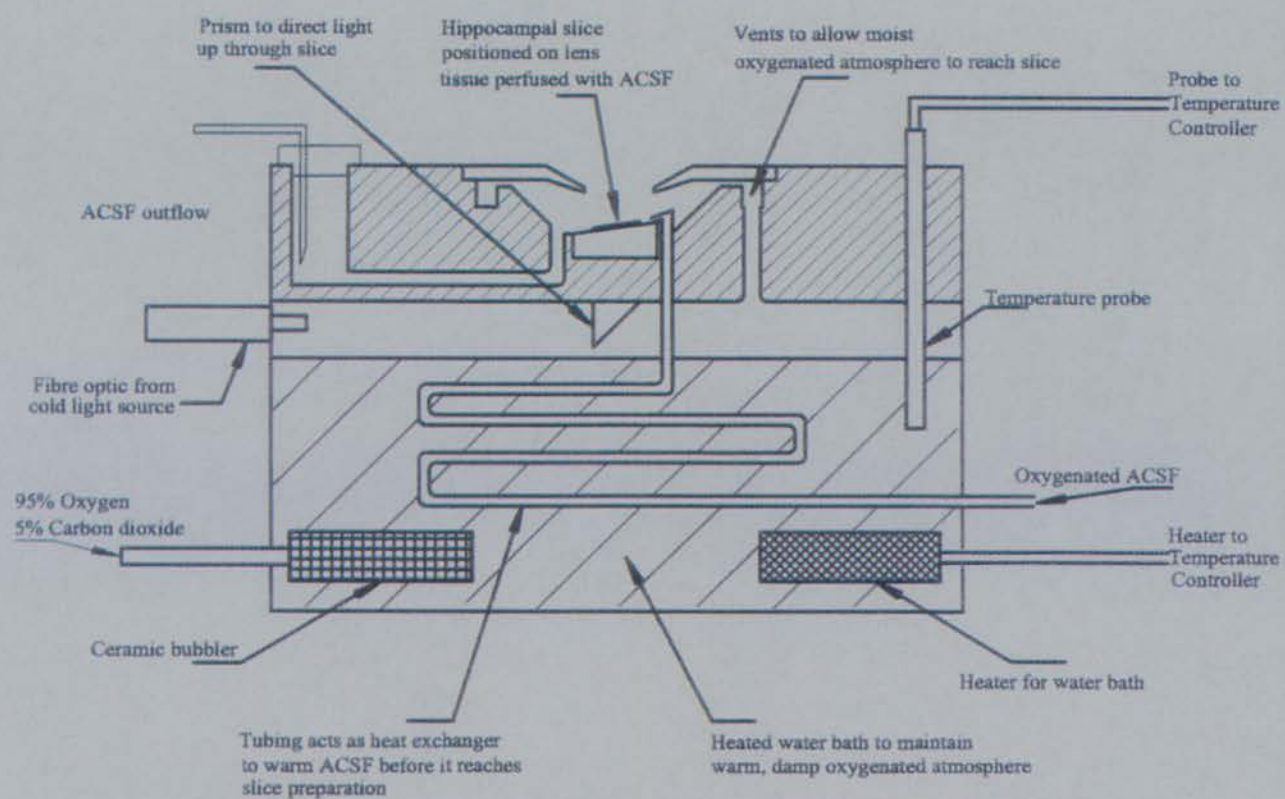


Figure 2.2 Photographs of slices in the recording chamber during an experiment.

- a. The position of the electrodes within the recording chamber. The recording electrode (Rec.) is to the right, and the two stimulating electrodes are on the left (S1 and S2 respectively).
- b. Slices shown during the preincubation period. Note that the lens tissue (i) is wet, although the fibres of the tissue are still clearly visible indicating the necessary level of aCSF in the chamber for long-term recordings. If the tissue became 'waterlogged' the stability of recordings was severely compromised. Mechanical stability and a constant level of aCSF were achieved with slow flow rates of $\sim 150 - 200 \mu\text{l}/\text{min}$. The aCSF was perfused from a needle (iv) resting at the edge of the chamber insert. Drainage was aided by the aCSF flowing along a channel made by the second 'pole' stimulating wire (iii) and the additional positioning of two small pieces of wire (ii) in the chamber.
- c. The entire rig was insulated, and a heater underneath the air table allowed for constant temperatures at $\sim 32^{\circ}\text{C}$ to be maintained throughout recordings.

a.



b.



c.



2.1.5 Problems associated with condensation and mechanical instability.

It is critical for any sort of LTP experiment, but even more so for L-LTP recordings, that neither the tissue nor electrodes move during the course of the experiment. Small changes in the position of the stimulating or recording electrodes can lead to changes in the EPSP profile. Movement may result from condensation building up on the electrodes; the additional load may cause them to move. What is more likely, and traumatic to the slice, is if condensation builds up on the electrode and then falls onto the surface of the slice. The sheer mechanical force and the potential change that this may have on the pH of the surface of the slice are extremely detrimental to the stability of recordings. Two steps were taken to minimise these factors. Firstly, sheathing the electrodes in glass capillary tubing strengthened them and so helped minimise movement. Secondly, the adjustments discussed above aimed at keeping the temperature constant all greatly improved the stability of recordings as droplets were seen on electrodes only very rarely.

2.1.6 Recording and measurement of evoked responses.

The great advantage of the hippocampal slice preparation is that it is possible to visualise the presynaptic fibre tracts that innervate the postsynaptic neurons from which recordings are made. Evoked field potentials were recorded postsynaptically in response to a stimulus applied to the presynaptic fibres. These recordings were made extracellularly; therefore they reflect the responses from ‘populations’ of neurons within the area stimulated. Recordings were made from the dendritic region of CA1 pyramidal cells, and stimulation was applied to the Schaffer-collateral/commissural pathway. The dendritic response is termed the ‘field excitatory postsynaptic potential’ or fEPSP. However, action potentials can also be recorded even when recording from the dendritic tree. The action potential or ‘population spike’ is recorded simultaneously and is superimposed upon the fEPSP. When recording from the stratum radiatum of CA1 cells, (as was always the case in the experiments reported here) the direction of the field-evoked potential is negative when a stimulus is applied to the Schaffer-collateral commissural pathway. This reflects an inward current associated with dendritic synaptic activity. In this configuration, it is also possible to detect a positive deflection on the rising phase of the fEPSP as stimulation intensity increases. This is due to the synchronous generation of action potentials in the postsynaptic cells in the recording region i.e. the population spike.

Field EPSPs were recorded with monopolar stainless steel electrodes with a 250 μM tip diameter, tapered at 12° , with a resistance of 5 $\text{M}\Omega$ (AM Systems Inc. USA). The reference electrode for recordings was made by connecting the ground wire to the suction needle, which was in constant contact with the aCSF. Field EPSPs were amplified and filtered at 1 Hz and 5 kHz (AM Systems Inc. USA). The initial slope of the field EPSP (measured by linear regression between two fixed time points), and the fEPSP amplitude were monitored on-line by an Acorn A5000 computer running specialised software (Roger Spooner). The sampling rate was 10 kHz.

For stimulation, the same monopolar stainless steel electrodes were used. The timing and duration of test stimuli (biphasic pulse, pulse half-width 100 μsec , ranging from 10-100 μA .) were controlled using a Neurolog system. As the stimulating electrodes were monopolar, the 'second pole' of the electrode was a piece of chlorided silver wire that sat in the outflow channel of the chamber (one of the 'wicks' as described in Section 2.1.3). This configuration was chosen to achieve maximal activation of presynaptic fibres. The stainless steel electrodes provided good mechanical stability throughout the course of long-term recordings; the small tip diameter minimises tissue damage and allows for easy positioning of a number of electrodes within the same area of the slice.

For all experiments, the maximum evoked response was calculated by conducting an input/output curve (I/O curve) after the pre-incubation period and before the baseline recording session started. Two stimulating electrodes were positioned in the Schaffer-collateral pathway (stratum radiatum) each positioned approximately 2 mm apart to stimulate independent synaptic populations. Single biphasic pulses (100 μs half-width) were applied to each stimulating electrode in succession, once every 30 s, at intensities ranging from 10-100 μA . The maximum response was, in most cases, elicited with a pulse at an intensity of 80-90 μA . Due to limitations of the software used it was not possible to test for independence of the pathways using a paired pulse protocol, whereby individual stimuli are delivered to each pathway within a short (50-100 ms) interval of each other. The main test of independence used therefore was post-tetanic potentiation in the untetanised pathway following delivery of a tetanus to the second pathway (described in detail in results sections of later chapters).

For test pulse stimulation, the intensity was set to evoke a response that had a slope (mV/ms) that was ~50 % of the maximum slope obtained during the I/O curve. At this half-maximal level, the slope of the EPSPs recorded was in the range of 1.5 to 2.5 mV/ms (the amplitude being ~2 - 3 mV). During both the pre-tetanus baseline and post-tetanus periods, each pathway received a test stimulus once every 2 min, with stimuli to the two pathways interleaved at 1 min intervals. If the slope of the fEPSP recorded for each pathway increased or decreased by > 30 % during baseline recording, stimulation was paused and the test intensity was either increased or decreased to a level which elicited a half-maximal response. If in any given experiment, the time taken to achieve a stable 1 hr period of baseline recording exceeded 3 hr, the experiment was terminated and, if possible, recordings began with another slice. Generally, slices in which it took a long time to achieve a stable 1 hr baseline period did not produce stable long-term recordings post-tetanus.

2.3 Behavioural analysis.

The watermaze (Morris et al., 1981, 1984) is an open-field navigation task. It is spatial in nature and very sensitive to disruption of hippocampal function (Morris et al., 1982). The aim of the 'standard' reference memory version of the watermaze is for the rat to find a hidden platform by swimming through the open-field. The platform constitutes the only escape that the animal has from the water. As there are no intra-maze cues, the rat has to learn the importance of the extra-maze cues and how they relate to the platform's location. These cues then become the means for allocentric navigation.

Animals used for behavioural testing were adult male Lister Hooded rats (250 – 500 g). Animals were housed in groups of 2 per cage, and were maintained on a 12:12 hr light:dark cycle, with lights on at 08:00 hr. Food and water was supplied ad libitum. I am grateful to Andrew Bernard and Sharon Rossiter for their care of the animals.

2.3.1 The watermaze.

The watermaze is a pool 2 m in diameter and 60 cm in height (Figure 2.3). It is made of fibreglass, the interior is coated with gelcoat and painted white. It sits 60cm above the floor level

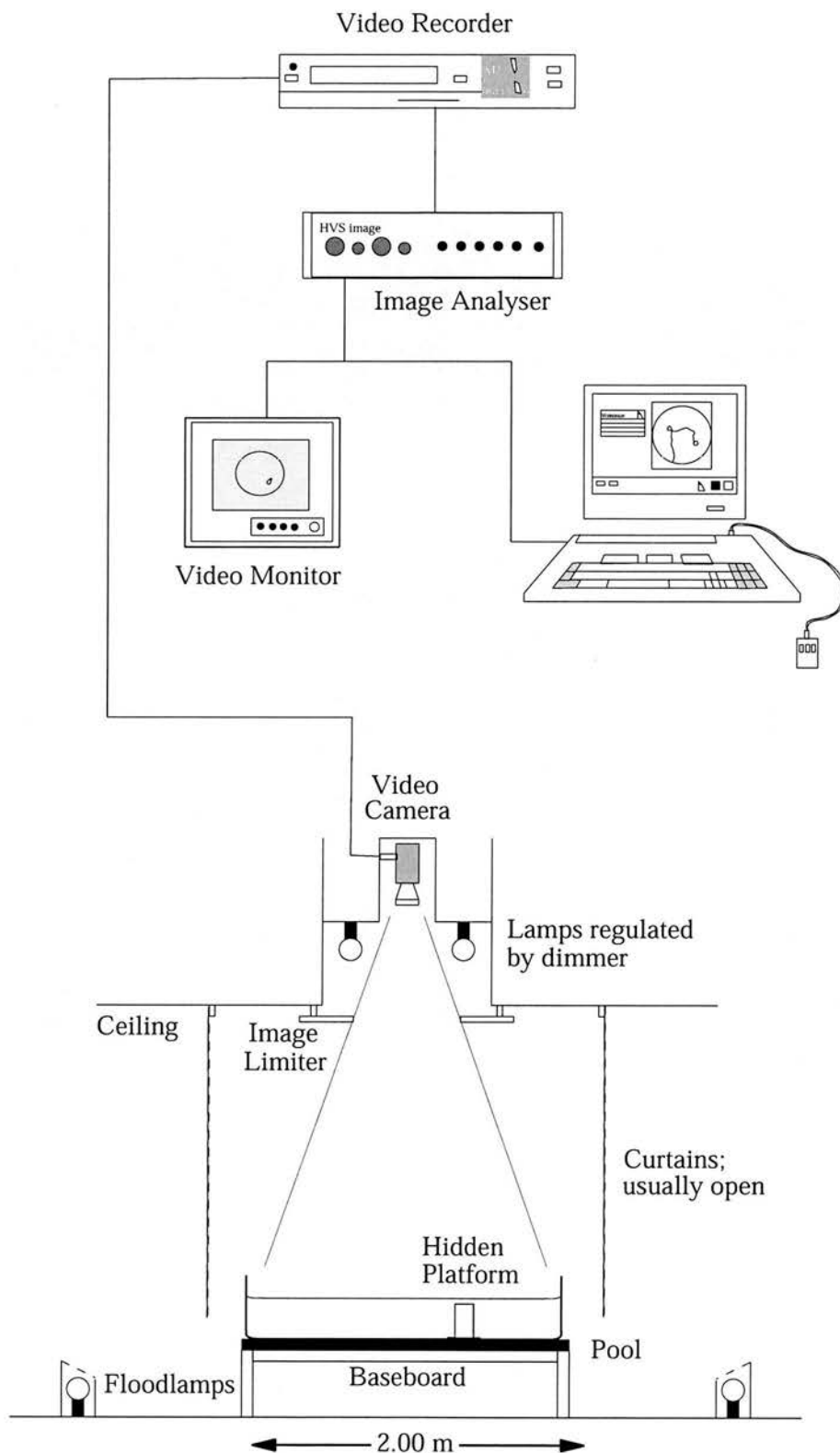
on a frame in the middle of a room with prominent extra-maze cues such as wall posters, cabinets and metal racks. The water reaches a level of approximately 40 cm. Escape is onto a solid platform, 11cm in diameter, whose surface is only 1-2cm below the water's surface. The water is made opaque by adding white latex (Cempolatex Ltd.). This makes the submerged platform invisible for a swimming rat. The rat's path is recorded by a recessed video camera overlooking the pool and the video signal is relayed to a video recorder. This signal is fed to an image analyser (HVS VP112). The co-ordinates of the rat's path are sampled at 10Hz by an Acorn computer running the 'Watermaze' software (Roger Spooner). The programme can obtain different measures such as the time taken by the rat to find the platform (escape latency), the path length, the percentage time spent near the side-walls, the swim speed, or the percentage time spent in a specified area of the pool.

2.3.2 The general protocol.

The water is maintained at $25 \pm 1^\circ\text{C}$. In a normal trial, the rat is placed in the water facing the walls at any four prearranged positions corresponding to the four cardinal points (North, South, East and West). The rat then swims in search of the platform. The rat is left on the platform for 30sec to allow it to look around and associate the platform position with extra maze cues. If the rat does not find the platform in 120 s it is guided to it. This rarely involves physical interference with the rat, as placing one's hand above the location of the target is usually sufficient. The platform is a metal cylinder (11cm in diameter, 27cm in height). It is fixed and stands just 1-2cm below the water level. During a trial the rat cannot see where the platform is, but may 'bump' into it while swimming in the pool. The opportunity therefore arises to climb onto the platform and 'escape' from the pool. In a very standard watermaze experiment, a 'normal' daily procedure will consist of a session of four consecutive trials per rat. At the end of each trial the rat is dried with a towel and placed in a cage until the next trial. At the end of a session of trials, the rat is dried, placed in a cage and taken back to the animal room and left under a heat lamp until completely dry.

Figure 2.3 The watermaze apparatus.

The pool is located in the centre of a room containing prominent extra-maze cues. The room is diffusely lit by floodlights placed in the 4 corners. In order to obscure the submerged platform, the water was made opaque by the addition of cemoplatex liquid (Cemoplatex Ltd). A recessed video camera was placed directly above the centre of the pool to monitor the paths taken by the animals during swimming. The video signal was relayed to a video recorder for both on- and off-line analysis and fed to an image analyser (HVS VP112). Co-ordinates were sampled at 10 Hz by an Acorn Archimedes 3000 computer running the “Watermaze” software package written by R. Spooner.

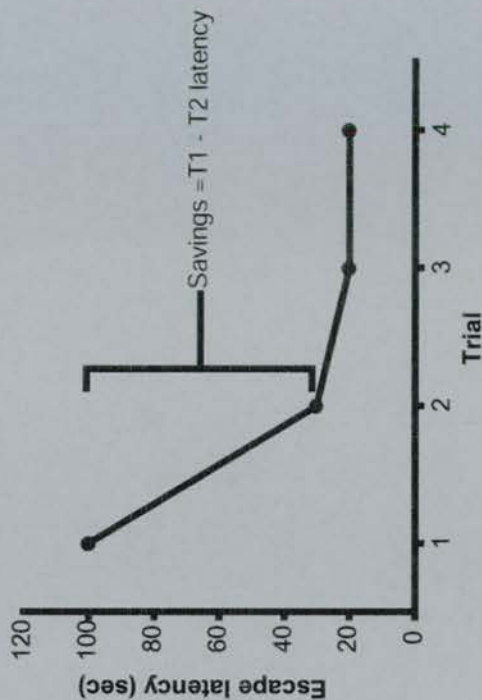
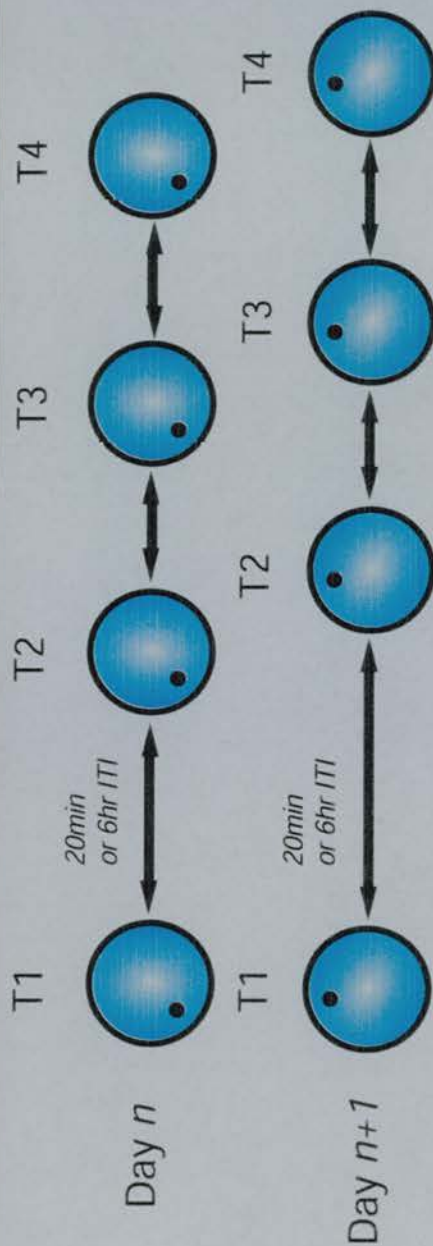


2.3.3 *The delayed-match-to-place task.*

In this version of the watermaze, originally described by Steele & Morris, (1999) a rat receives four trials per day to learn a new platform position each day. The platform position is changed quasi randomly from day to day (Figure 2.4). This means that on the first trial of any day (Day n), the rat has no information to suggest where the platform is located. On subsequent trials (trials 2-4), the location of the platform remains the same as that on trial 1 of that day. On subsequent days (Day $n+1$), the platform moves again on trial 1, and remains fixed in that position for the remaining trials. Therefore, on any day, performance on trial 2 is a measure of the rat's memory for the platform's position on the first trial (i.e., a one-trial memory test). Normal rats show poor performance on trial 1 of any given day, but with training, a significant improvement occurs on trial 2 with little further improvement on the subsequent trials.

Figure 2.4 The delayed matching-to-place watermaze task.

The Delayed Matching-to-Place (DMP) Watermaze Protocol: Animals are given 4 trials/day (T1-T4) with the hidden platform staying in the same location for a given day. The platform moves location between days. On any given day (Day n , Day $n+1$), the delay between trials 1 and 2 is varied. Subsequent trials (trials 2-4) have the same inter-trial interval (ITI).



Escape latency is long on trial 1 of a given day, but improves rapidly on trial 2, without any greater improvement on subsequent trials. Savings is calculated as the decrease in escape latency between trials 1 and 2.

2.4 Surgical Procedures.

2.4.1 Implantation of intra-hippocampal cannulae.

For the experiments reported in Chapter 7, Dr. Johan Sandin carried out all the surgical procedures. The microinfusions were carried out by Dr. Stephen Martin. I am grateful for their contribution; I conducted the behavioural aspect, 'blind' with respect to the drug administered.

Intra-hippocampal cannulae were implanted into each hippocampus in order to deliver the dopamine D1/D5 receptor antagonist into the hippocampus. Rats were anaesthetised with Avertin (a tribromoethanol-based anaesthetic) given intra-peritoneally at a dose of 10 mg/kg while under the effect of halothane gas. The top of the head was shaved and cleaned with alcohol. The animal was then placed in a Kopf stereotaxic frame with the mouth bar placed such that bregma and lambda co-ordinates would rest in the horizontal plane. An incision was made longitudinally in a line mid-way between the ears. The skin was held at the sides with forceps exposing the top of the skull. Bregma co-ordinate was taken and compared with lambda. The co-ordinates for implantation were calculated from bregma and marked over the skull. A dental drill was used to remove the bone overlying the implantation points (Drill hole diameter = 0.7 mm). Care was taken not to damage the dura during the drilling and to regularly cool down the area with sterile saline to prevent the heat from the drill from damaging the cortex. The animals were implanted bilaterally with permanent 26 gauge steel guide cannulae (26 gauge, diameter = 0.45 mm, length = 3.4 mm) using the stereotaxic frame. The coordinates were based on the stereotaxic plates of the Paxinos and Watson atlas; anterior-posterior coordinate -4.5 mm refers to bregma, lateral coordinate ± 3.0 mm to the midsagittal suture line, and ventral coordinate -3.9 mm (injection site) to the surface of the skull. The guide cannulae were secured to the skull with dental cement and miniature screws. The skin was sutured around the head cap and the wound covered in aureomycin. A dummy cannula was put into the guide cannula and the rats were given at least 7 days of recovery in the home cage.

2.4.2 Microinfusions.

A microinfusion pump was used for bilateral infusion (1 µl/side), at a flow rate of 0.2 µl/min over five minutes. Syringes (5 µl) were mounted on the pump and connected with injection cannulae (33 gauge, diameter = 0.2 mm, length = 3.9 mm) using flexible polyethylene tubing. During infusion, the animal was free from any restraint. The injection cannulae were left in the guide cannulae for two minutes after infusion to avoid backflow. After injection the dummy cannulae were placed back in the guide cannulae. Behavioural testing began 15 min after the infusion.

2.5 Statistical analysis.

All statistical analyses were performed using SPSS computer software. For the analysis of the electrophysiological recordings and the delayed-match-to-place behavioural experiment, three main statistical tests were performed. The first involved repeated measures analysis of variance (ANOVA). In order to compare pathways receiving high frequency tetanisation to an independent control pathway, paired samples t tests were performed. When multiple paired t test comparisons were carried out the p values were adjusted according to Bonferroni correction for multiple comparisons i.e., the p values for each comparison were multiplied by the number of individual t tests conducted. The final statistical comparison used was one-samples t tests when measurements of post-tetanic potentiation were conducted. This involved a comparison whereby the normalised response after a high frequency tetanus from each pathway was compared to the baseline level of 100 %. Further details of statistical analyses are provided in the individual results chapters.

Chapter 3. Induction of late-phase LTP (L-LTP).

3.1 Introduction.

Most of the published literature concerning LTP is based on recordings that typically last, at the most, ~2 - 3 hr. It is not the case that the study of L-LTP involves nothing more than allowing the experiments to run for longer. There are specific obstacles to overcome in order to record stable LTP that lasts for periods of 6 hr or more. As was discussed in Chapter 2 of this thesis, a large proportion of the project involved the initial development and refinement of *in vitro* electrophysiological techniques required for long-term recordings. In order to reach the stage where stable recordings were possible for extended periods (a time when the experiments described in this chapter commenced), many pilot studies were necessary. Some of examples of 'unsuccessful' experiments are presented at the end of this chapter, but the primary focus is on the findings secured once L-LTP was routinely established.

3.1.1 L-LTP requires multiple high frequency tetanic trains.

The experiments discussed in this chapter detail the first steps towards determining the induction requirements of L-LTP with high-frequency stimulation. Many people have considered the type of tetanic stimulation protocol used as the basis for differentiating between the stages of LTP. For example, Huang et al., (1994) concluded that a single train of 100Hz for 1 s induces E-LTP, i.e. LTP that persists for 1-3 hr. More persistent, L-LTP requires repeated tetanisation.

In the L-LTP literature, tetanus protocols of multiple high frequency tetanic trains are used to induce long-lasting LTP; consisting of three to four trains of 100 Hz stimulation (1 second duration) with an inter-train interval of 5-10 min (Frey et al., 1988; Huang and Kandel, 1994; Huang et al., 1994; Nguyen et al., 1994; Huang et al., 1996; Impey et al., 1996; Abel et al., 1997; Frey and Morris, 1997; Baranes et al., 1998; Winder et al., 1998; Lu et al., 1999; Scharf et al., 2002). It is widely held that the main difference between the induction of E-LTP and L-LTP is that repeated tetanisation with high frequency trains separated temporally is necessary in order to see the sustained potentiation at 8-10 hr post-tetanus that characterises true L-LTP, whereas E-LTP does not have this requirement. However, multiple trains of 100 pulses are not the only way of achieving L-LTP. For example, 4 trains of 20 pulses at 100 Hz with a 6 s

inter-train interval (ITI) induced L-LTP in the CA1 of rat hippocampal slices (Wong et al., 1999). In mice, 15 trains of 4 pulses at 100 Hz with an ITI of 200 ms (Nguyen and Kandel, 1997) or 3 trains of 30 pulses at 100 Hz with a 5 s ITI (Korte et al., 1998) can induce L-LTP.

3.1.2 Other factors contributing to L-LTP.

Many other factors contribute to whether or not successful L-LTP is induced including the 'health' of the slices, the duration of the initial incubation period, and various features of the slice chamber design. One notable factor is that as these experiments are conducted at 32 °C, condensation builds up on the electrodes. If there is significant build up of condensation, droplets of water may drip onto slices at various time-point in the experiment disrupting the immediate microenvironment of the slice. Heating the entire Faraday cage environment to 32 °C prevented this problem and enabled long-term overnight recordings.

3.1.3 Aim of experiments.

Experiments in this chapter aimed to replicate the basic findings of Frey and Morris, (1997). It was important to establish that LTP could be induced that persisted for at least 5 - 6 hr post-tetanus, and that the potentiation was input specific (i.e., homosynaptic), in order to address some of the questions that were central to this thesis. Similar tetanus protocols were used to those of Frey and Morris, (1997, 1998a) i.e., high-frequency tetanic stimulation delivered at 10min intervals. During the tetanus, the duration of each individual pulse is broadened to twice that of the test pulses (i.e., biphasic pulses 200 μ s per half pulse during each tetanus, 100 μ s per half pulse during test stimulation). This is done in order to ensure maximal stimulation and the recruitment of many fibres during the tetanus.

3.2 Methods.

3.2.1 Preparation of slices, and recording set-up.

Experiments were conducted with hippocampal slices prepared from 7-8 week old male Wistar rats (see Figure 3.1. for schematic). Slices (400 μm) were prepared as described previously (see Chapter 2) and maintained at 32 °C in an interface recording chamber. Slices were continuously perfused with aCSF and allowed at least 2.5 hr to equilibrate before electrodes were positioned. The position of the electrodes within each slice is schematically illustrated in Figure 3.1. With this configuration it was possible to evoke responses from two independent but overlapping input pathways (S1 and S2 respectively) and record the extracellular field EPSP response to stimulation of each pathway. The independence of each pathway was established by determining whether there was any post-tetanic potentiation (PTP) in the non-tetanus control pathway following high frequency stimulation of the tetanus pathway.

3.2.2 Tetanus protocol.

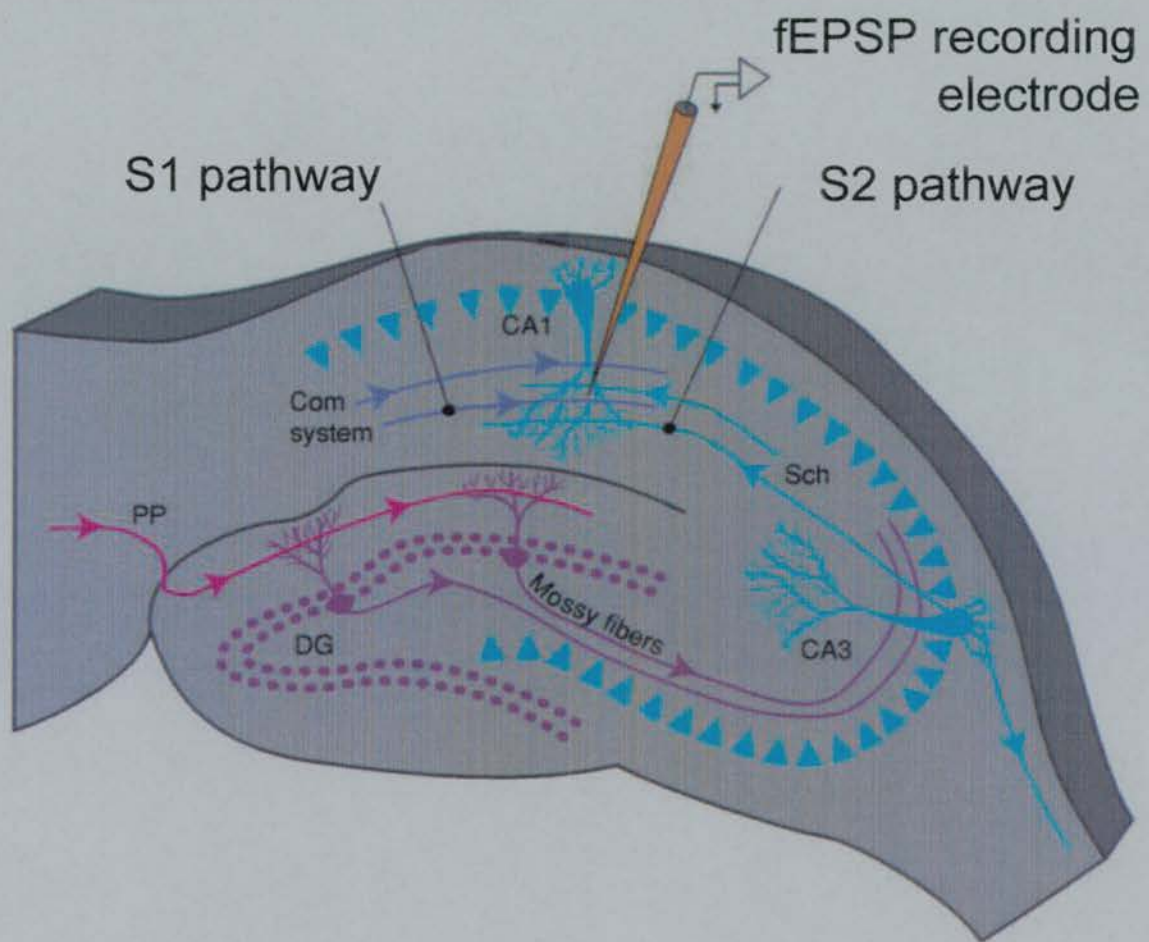
For the induction of L-LTP, a tetanic stimulation protocol which consisted of three high-frequency tetanic trains at 100 Hz in 1 s burst, given three times with an inter-train interval of 10 min (biphasic pulses with 200 μs per half-wave i.e. twice the duration of test stimulation) was used.

Drift in the magnitude of potentials was occasionally observed. It was essential to establish a criterion for inclusion in the overall data set. Thus, experiments in which the drift was $\geq 30\%$ either upwards or downwards were excluded. Recordings were made for a minimum of 6 hr post-tetanus, thus a drift of $\leq 30\%$ corresponds to a change of $\leq 5\%$ per hour.

Figure 3.1 Schematic representation of the in vitro hippocampal slice and the position of the electrodes with CA1.

Hippocampal slices (400 μm) were prepared and incubated in an interface recording chamber at 32 degrees for ~ 2.5 hr prior to placement of electrodes. Two stimulating electrodes (S1 pathway and S2 pathway, respectively) were positioned within the stratum radiatum of the CA1 region, either side of an extracellular recording electrode as shown. The distance between the electrodes was ~ 2 mm, in order to maximise the chances of stimulating two independent, but overlapping, pathways in the Schaffer-collateral commissural area.

Abbreviations; *Com system* = Schaffer-collateral commissural fibres. *CA1* = Cornu Ammonis area 1. *CA3* = Cornu Ammonis area 3. *DG* = dentate gyrus. *PP* = perforant pathway.



3.3 Results.

3.3.1 Assessment of maximal response (input/output measurement).

After positioning of the electrodes within the slice, the maximal output of each pathway was determined by conducting an input/output curve. Test stimuli (biphasic square-wave pulses 100 μ s per half-wave were delivered to each pathway once every 30 s), were applied to both S1 and S2 paths at a range of stimulus intensities (from 10 - 90 μ A in 10 μ A steps). The averaged response over this range of stimulation intensities increased from 0.32 to 3.27 mV/ms for S1, and from 0.29 to 3.05 mV/ms for S2. Statistical comparison of the twelve slices showed no difference between pathways in the response to stimulation across all intensities used [$F > 1$]. The average values for the input/output measurement are shown in the inset of Figure 3.4.

Test stimuli (a biphasic pulse 100 μ s per half-wave, delivered to each pathway once every 2 min) were applied to both S1 and S2 pathways. The baseline test stimulation intensity was set as the stimulus that evoked a response that was 50 % of the maximum response measured during the input/output curve. A 1 hr period of baseline recording was then conducted. If either pathway showed >30 % drift upwards, or downwards in the response, the experiment was paused and new stimulation intensities were chosen which again elicited a 50 % of maximum response. If the response of either pathway was unstable (even after resetting the stimulation intensities), for 2 -3 hr the experiment was terminated. One pathway was designated the 'tetanus' pathway and received tetanic stimulation; the 'control' pathway received test stimulation for the entire duration of the experiment.

3.3.2 Stable baseline responses, and analysis of post-tetanic potentiation (PTP).

In all experiments, stable baseline responses were recorded for 1 hr prior to high-frequency tetanisation from two independent inputs in the stratum radiatum. Control experiments were conducted (these were interleaved with the experiments where high frequency stimulation was delivered to one pathway), in order to determine the stability of two independent, non-tetanised pathways during long-term recordings. These experiments (n=2), demonstrated that the necessary technical requirements were established for very stable long-term recordings in CA1 hippocampal slices (see Figure 3.2).

Representative field EPSPs are shown from an individual L-LTP experiment in Figure 3.3. Note that the profile of the response is marginally different for each pathway. The distance between the stimulating electrodes (i.e., approximately 2 – 3 mm) was such that each electrode is stimulating different, but potentially overlapping, inputs to the same group of CA1 pyramidal cells.

However, the main test for the independence of the two pathways used was measurement of the post-tetanic potentiation, (PTP) in both pathways. PTP was measured for 4 min after the first of the three high-frequency trains used to induce LTP (i.e., as test stimuli were taken at 2 min intervals, the responses measured to assess PTP were the first and second fEPSPs recorded immediately after the tetanus). If the pathways are independent, PTP should not be observed in the ‘control’ (S2) pathway following tetanus to the ‘tetanised’ pathway (S1). Statistical comparison with a one-sample t-test where the normalised response from each pathway was compared to the baseline level of 100 %, showed that there was no PTP in the control pathway after delivery of the first of the three tetani (fEPSP Slope = $101.7 \% \pm 3.3$ s.e.m., $t = 0.52$, $p > 0.62$) compared to the baseline level. The tetanised pathway showed robust PTP (fEPSP Slope = $234.2 \% \pm 17.0$ s.e.m., $t = 7.86$, $p < 0.001$).

Figure 3.2 Stable baseline responses from two independent input pathways in CA1.

Experiments ($n = 2$) where two independent inputs (S1 and S2) received low frequency test stimulation, in order to determine the baseline stability during long-term recordings in vitro. Each pathway received test stimulation once every 2 min, this alternated between S1 and S2 for the entire duration of the experiment. Responses were normalised to the first 60 min period. Error bars indicate \pm s.e.m. Symbols: S1 pathway ●. S2 pathway ○.

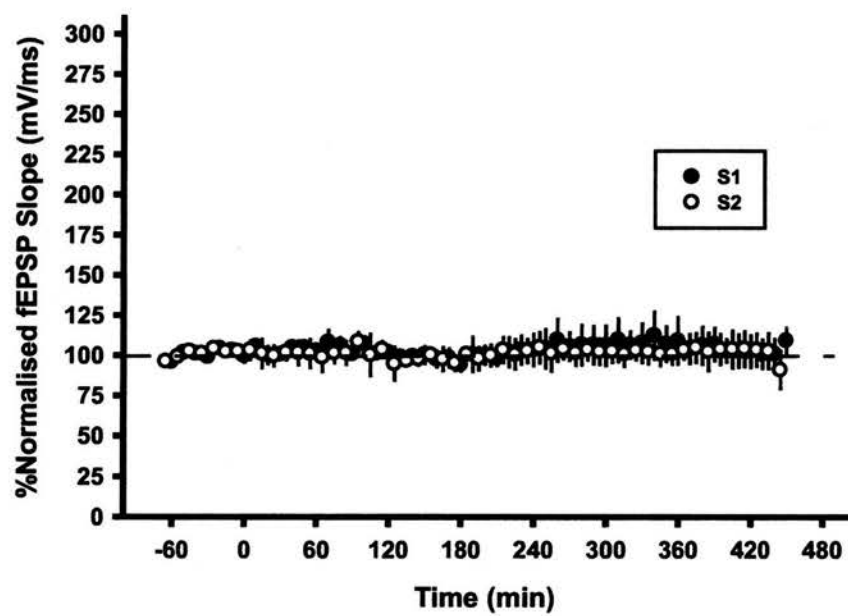
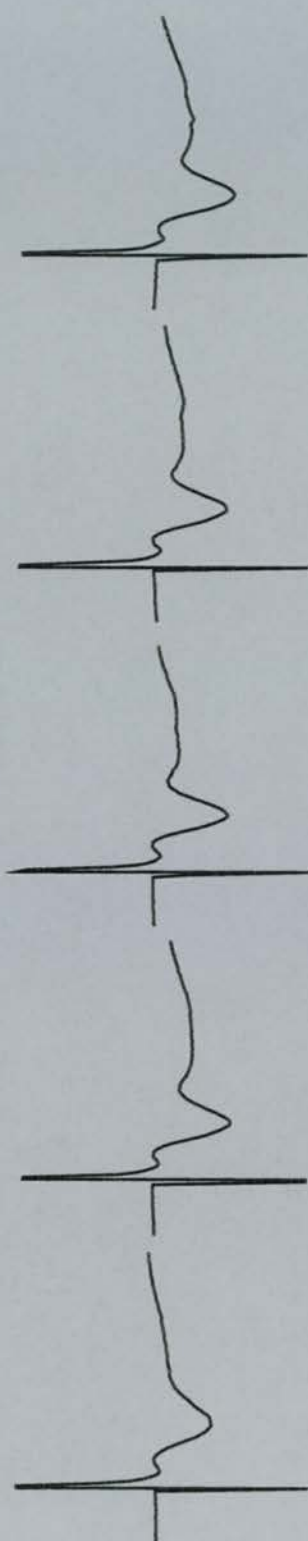


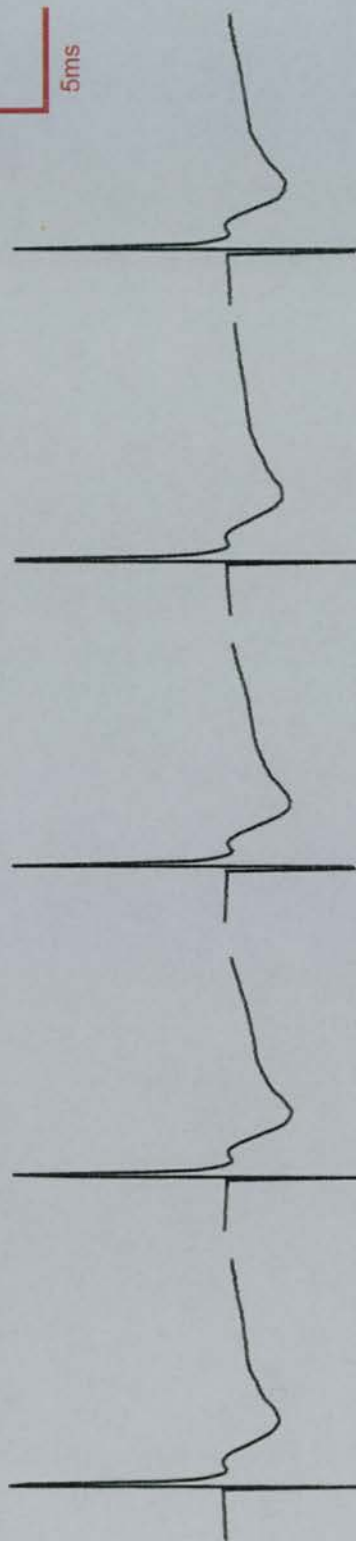
Figure 3.3 Representative example of fEPSP waveforms from an individual experiment with high frequency stimulation to induce L-LTP.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 3.4 a.

S1 - Tetanised pathway



S2 - Non-tetanised pathway



360 min
post-tetanus

180 min
post-tetanus

60 min
post-tetanus

10 min
post-tetanus

30 min
pre-tetanus

3.3.4 The induction of L-LTP in hippocampal CA1 area.

An individual example of one of the experiments, which form part of the grouped data presented later, is shown in Figure 3.4 a. The tetanus (i.e. 3 x100 Hz, 10 min inter-tetanus interval) was delivered to the S1 pathway, 10 min post-tetanus the response was 188.2 % of the pre-tetanus baseline level. The level of potentiation was 176.5 % 60 min post-tetanus, this declined to 162.0 % at 180 min, but remained at a stable level of 158.9 % at 360 min post-tetanus. The control pathway (S2) showed no change at any of these time points.

The results of the L-LTP experiments are shown in Figure 3.4 b. For statistical analysis and graphical representation, the responses were averaged across 10 min periods (i.e. a total of 5 successive responses for each pathway) for each experiment. For each successive 10 min time point (i.e. bins of 5 responses) the mean, standard deviation, and standard errors were calculated for all the experiments (n = 12).

Input-specific L-LTP was clearly induced in these experiments, justification for this statement comes from the following analysis. A repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors was conducted. There was a significant difference between the two pathways [$F(1, 11) = 112.6$; $p < 0.001$], indicating that the tetanus produced robust L-LTP. A significant pathway x time interaction was also found [$F(3, 33) = 23.2$; $p < 0.0005$]. Subsequent analysis revealed that the decline in response across time was due to a small decrease in the tetanised pathway (S1) [$F(3, 33) = 18.8$; $p < 0.001$]. There was some decline in the potentiation during the first 2 – 3 hr post-tetanus. This subsequently stabilised, with no subsequent reduction at later times, and there was no significant change in the control pathway (S2) for the entire post-tetanus period [$F < 1$].

In summary, the tetanised pathway (S1) showed robust potentiation after the three high-frequency tetanic trains. This LTP subsequently declined but was still significantly greater compared to the control pathway (S2) at 360min. The non-tetanised control pathway (S2) remained very stable for the entire post-tetanus period. The averaged values \pm s.e.m. are presented in Table 3.5. Statistical comparisons (Paired samples t tests with Bonferroni correction for multiple comparisons) between the tetanised and the control pathways at various time points during the experiment (Figure 3.4 c) showed significant differences at all times.

Figure 3.4 Induction of L-LTP in CA1 of the hippocampal slice.

- a. An individual experiment showing a representative example of the induction of L-LTP (the waveforms in Figure 3.3 are from this experiment).
- b. Grouped data for experiments in which L-LTP was induced ($n = 12$). For all experiments, the electrodes were positioned within the stratum radiatum. Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. Baseline (100 %) was taken as the mean of the values in consecutive 10 min periods (i.e. 5 successive evoked responses), and 60 min of pre-tetanus baseline response was recorded before three 100 Hz trains (indicated by arrows) were delivered to S1 at 10 min intervals. Error bars indicate \pm s.e.m. Symbols: S1 tetanised pathway ●. S2 non-tetanised pathway ○.
- c. Statistical comparisons were made between the tetanised pathway (S1) and the non-tetanised control pathway (S2) after the tetanus at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at each time point. *** indicates a statistically significant difference with $p < 0.005$ (paired students t – test, with Bonferroni correction for multiple comparisons). Significant differences were observed at all times.

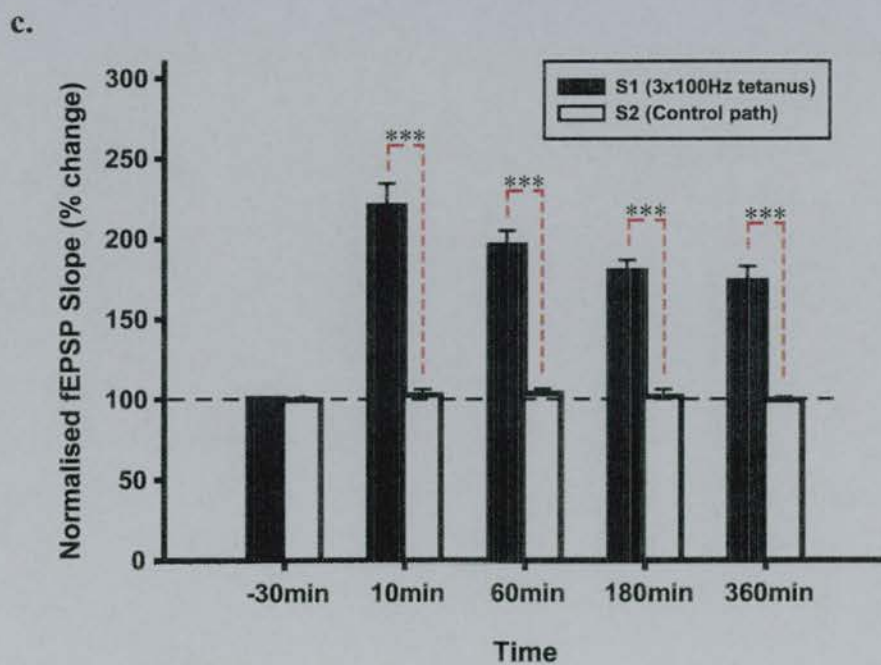
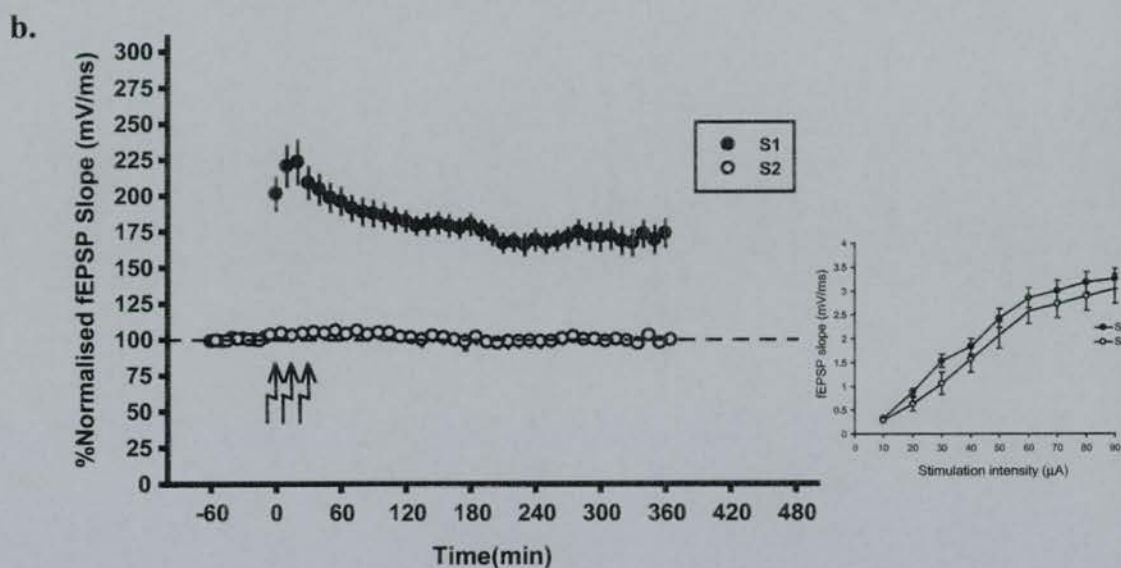
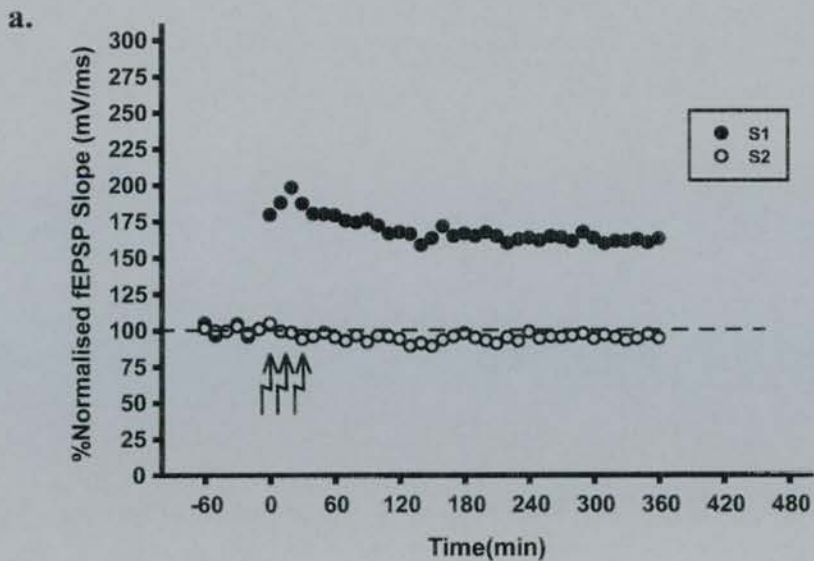


Table 3.5 Summary of statistical comparisons for L-LTP experiments.

Time (min)	S1 pathway normalised EPSP slope	S2 pathway normalised EPSP slope	t value	
-30	100.61 ± 0.71	99.81 ± 1.20	0.57	p > 0.1
10	220.62 ± 14.06	102.83 ± 3.58	9.55	p < 0.005
60	196.00 ± 9.16	103.63 ± 2.89	10.47	p < 0.005
180	179.85 ± 6.90	101.63 ± 4.63	9.20	p < 0.005
360	171.35 ± 9.76	100.11 ± 2.27	8.48	p < 0.005

Table 3.5: Paired samples t – test comparisons of S1 and S2 pathways, at a number of times before and after high frequency stimulation. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

3.5.1 Factors which influence the stability of long-term recordings and/or the persistence of LTP *in vitro*.

Many reports have been published that emphasise the various requirements necessary for preparation and maintenance of healthy hippocampal slices *in vitro* (Skrede and Westgaard, 1971; Richards, 1981; Alger et al., 1984). The minimum conditions needed for these types of experiments have already been discussed in Chapter 2. However, most of these reports, and the vast majority of *in vitro* experiments, only last for short period's i.e., 1 - 2 hr. The results reported here show that it is possible to successfully induce homosynaptic L-LTP with high-frequency tetanisation, and that the response in a non-tetanised control pathway remains stable for up to 6 hrs post-tetanus in these experiments.

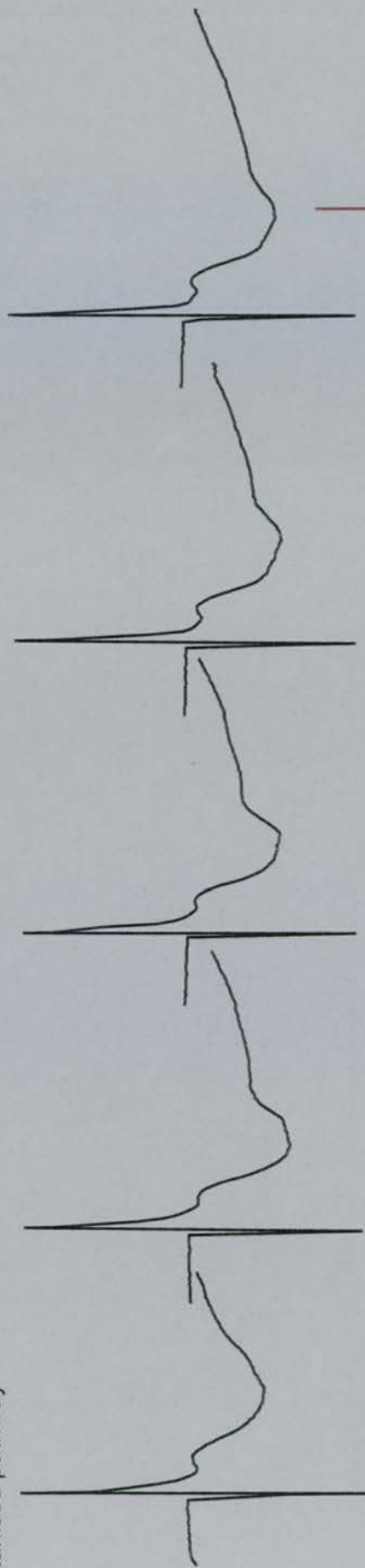
Temperature was considered as a likely major influence on the duration and/or the stability of long-term recordings. It is obviously a major goal of all *in vitro* experiments to try to approximate the conditions *in vivo* as near as possible. Therefore, being nearer more 'physiological' temperatures can be considered an important factor to be taken into consideration. At 'low' temperatures i.e. 27 °C, Thompson et al., (1985), found that calcium release may be compromised, resulting in abnormally prolonged after-hyperpolarizations. Another often cited effect of temperature relates to the effects of nitric oxide synthase (NOS) inhibitors on LTP induction in the slice. Williams et al., (1993) found that the ability of NOS inhibitors to prevent LTP induction occurs only at lower temperatures (24 °C) and not under more physiological temperatures (30 °C). This indicates that slices are sensitive to the temperature at which recordings are made. Whether this affects the quality, stability or persistence of LTP for long periods is another issue.

The results of experiments described above were obtained with the temperature of the recording chamber set at 32 °C. Experiments were also conducted with hippocampal slices at 28 °C. Examples of fEPSP waveforms from an experiment conducted at 28 °C are shown in Figure 3.6. These groups of experiments were interleaved across different days.

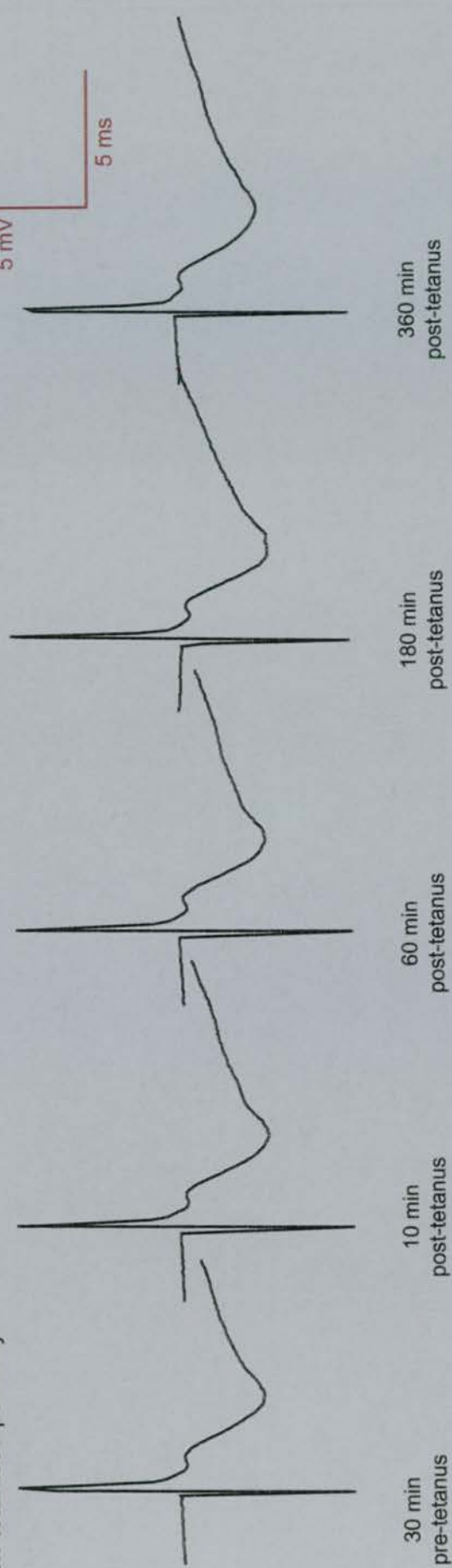
Figure 3.6 Representative example of fEPSP waveforms from an individual experiment conducted at 28 degrees.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms).

S1 - Tetanised pathway



S2 - Non-tetanised pathway



5 mV

5 ms

3.5.2 L-LTP at 28 degrees.

For statistical analysis and graphical representation, the responses were averaged across 10 min periods (i.e. a total of 5 successive responses for each pathway) for each experiment. For each successive 10 min time point (i.e. bins of 5 responses) the mean, standard deviation, and standard errors were calculated for all the experiments ($n = 6$).

Repeated tetanisation to pathway (S1) produced persistent L-LTP at 28 °C. This LTP subsequently declined but was still significantly greater compared to the control pathway (S2) at 360min. The non-tetanised control pathway (S2) remained stable over the entire post-tetanus period. A repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors was conducted. Figure 3.7 a, shows that there was a significant difference between the two pathways [$F(1, 5) = 89.8$; $p < 0.001$], indicating that the tetanus produced L-LTP. A significant pathway x time interaction was also found [$F(3, 15) = 3.6$; $p < 0.05$], as can be seen from the graph there was a significant decrease in the tetanised pathway (S1) over time [$F(3, 15) = 4.0$; $p < 0.05$]. There was no significant change in the control pathway (S2) during the post-tetanus period [$F(3, 15) = 3.2$; $p > 0.05$].

Statistical analysis (Paired samples t tests with Bonferroni correction for multiple comparisons) and averaged values \pm s.e.m. are presented in Table 3.8.

Figure 3.7 Recordings of L-LTP in the hippocampal slice at 28 degrees.

- a. Grouped data for experiments in which L-LTP was induced in the CA1 ($n = 6$), with the temperature of the recording chamber set at 28 degrees. Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. Baseline (100 %) was taken as the mean of the values in consecutive 10 min periods (i.e. 5 successive evoked responses), and 60 min of pre-tetanus baseline response was recorded before three 100 Hz trains (indicated by arrows) were delivered to S1 at 10 min intervals. Error bars indicate \pm s.e.m. Symbols: S1 tetanised pathway ●. S2 non-tetanised pathway ○.
- b. Comparison of L-LTP at either 32 degrees (●) or 28 degrees (○). Only the tetanus pathway from each experiment is shown.
- c. Statistical comparison of tetanus pathways from experiments at 32 degrees (filled bars) and experiments at 28 degrees (empty bars), at different times post-tetanus. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at each time point. * indicates a statistically significant difference with $p < 0.05$ (independent samples t – test, with Bonferroni correction for multiple comparisons). Significant differences were observed from 60 min and thereafter.

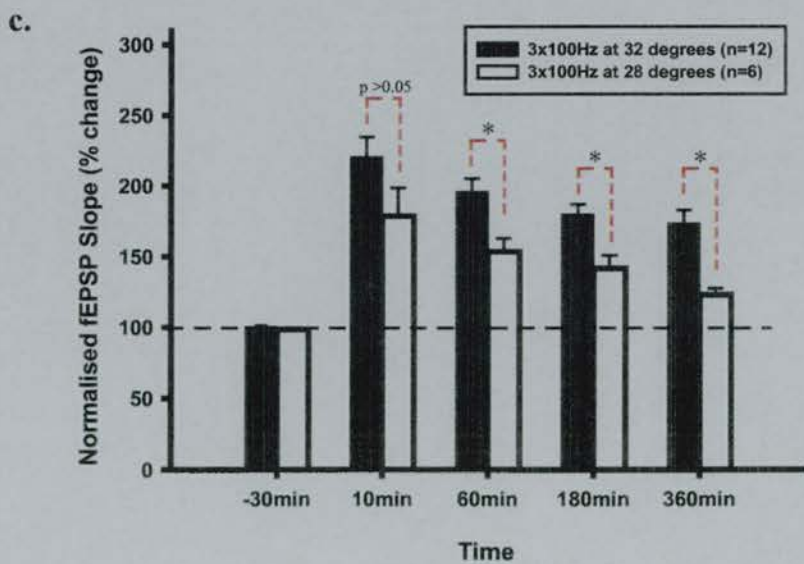
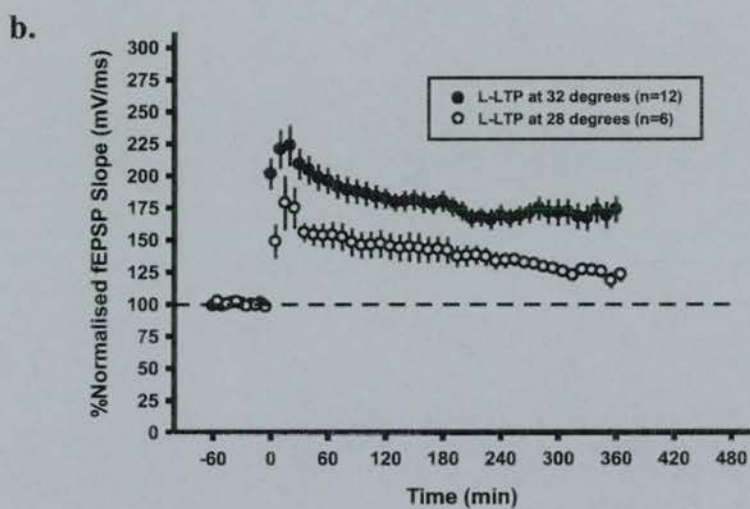
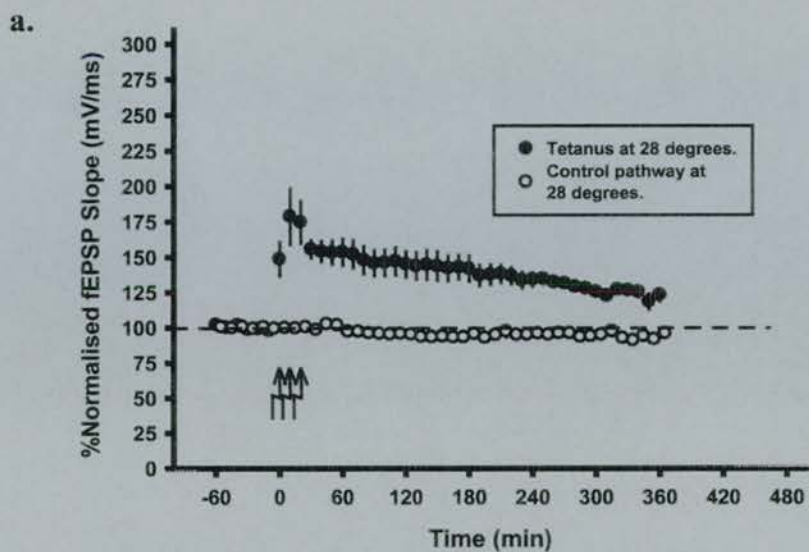


Table 3.8 Summary of statistical comparisons for experiments at 28 degrees.

Time (min)	S1 pathway normalised EPSP slope	S2 pathway normalised EPSP slope	t value	
-30	98.66 ± 1.12	99.37 ± 0.86	-1.38	p >0.05
10	178.66 ± 19.91	99.63 ± 1.80	4.16	p <0.05
60	153.53 ± 9.31	97.24 ± 2.64	8.02	p <0.005
180	141.68 ± 9.07	95.21 ± 1.51	5.92	p <0.01
360	123.04 ± 4.57	95.89 ± 1.92	4.54	p <0.05

Table 3.8: Paired samples t – test comparisons of S1 and S2 pathways, at a number of times before and after high frequency stimulation. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

3.5.2. L-LTP is temperature sensitive.

Comparison of experiments conducted at 32 °C with those conducted at 28 °C (Figure 3.7 b), suggested that less potentiation was obtained at 28 °C. This was confirmed by conducting the following analysis. The results are presented in Figure 3.7 c. A repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors and temperature of recording (28 and 32 °C) as a between-subjects factor was conducted.

Although there was no difference in terms of the duration of LTP induced at either 28 or 32 degrees, there was a significant difference between the magnitude of LTP at either temperature. There was a significant interaction between pathway and temperature at which the experiment was conducted [$F(1, 16) = 8.3$; $p < 0.05$] which suggests that the tetanus produced differential effects on the pathway that was tetanised depending on the temperature of the experiment.

As the interaction was significant, tests for main effects of each pathway were conducted with time (10 min, 60 min, 180 min, 360 min) as a within-subjects factor and temperature of recording (28 and 32 °C) as a between-subjects factor. The tetanised pathway was found to be significantly different between groups [$F(1, 16) = 9.1$; $p < 0.001$]; high frequency tetanisation produced greater LTP at 32 °C than at 28 °C. There was an overall decline in the tetanised path over time i.e., a significant effect of time [$F(3, 48) = 17.4$; $p < 0.001$], as can be seen from the graph (see Figure 3.8 b). However there was no differential degree of decline between experiments at 32 °C and those at 28 °C i.e., there was no significant time x temperature, interaction [$F(3, 48) = 0.2$; $p > 0.05$].

The control paths in both groups of experiments did not differ [$F < 1$], and there was no overall effect of time on the control paths for both experiments at 28 and 32 °C [$F(3, 48) = 0.5$; $p > 0.05$] suggesting that taking both groups of experiments overall, there was no difference in the control pathways.

Independent sample t tests confirmed that there were significant differences between the 28 °C and 32 °C experiments only at the later time points; potentiation during the first 10 min post-tetanus did not differ significantly between groups. These results are presented in Figure 3.7 c. and summarised in Tables 3.9 a & b.

Table 3.9 Statistical comparisons of experiments at 32 degrees compared with experiments at 28 degrees.

a.

Time (min)	S1 tetanus pathway for experiments at 32°C (normalised slope)	S1 tetanus pathway for experiments at 28°C (normalised slope)	t value	
10	220.62 ± 14.06	178.66 ± 19.91	1.72	p >0.05
60	220.62 ± 14.06	153.53 ± 9.31	2.91	p <0.05
180	196.00 ± 9.16	141.68 ± 9.07	3.26	p <0.05
360	179.85 ± 6.90	123.04 ± 4.57	3.36	p <0.05

b.

Time (min)	S2 control pathway for experiments at 32°C (normalised slope)	S2 control pathway for experiments at 28°C (normalised slope)	t value	
10	99.81 ± 1.20	99.63 ± 1.80	0.61	p >0.05
60	102.83 ± 3.58	97.24 ± 2.64	1.41	p >0.05
180	103.63 ± 2.89	95.21 ± 1.51	0.96	p >0.05
360	101.63 ± 4.63	95.89 ± 1.92	1.20	p >0.05

a. Independent samples t – tests for S1 tetanus pathways from experiments at 32 degrees and experiments at 28 degrees.

b. Independent samples t – tests for S2 control pathways from experiments at 32 degrees and experiments at 28 degrees.

For all comparisons, p values were adjusted according to Bonferroni correction for multiple comparisons.

3.6.1 The problems associated with stable in vitro recordings for long periods.

At times, difficulties in establishing the basic requirements for stable recordings were encountered. Although many successful experiments were carried out, the rate at which this was possible varied from ~ 1 out of 10, to ~ 3 in 5.

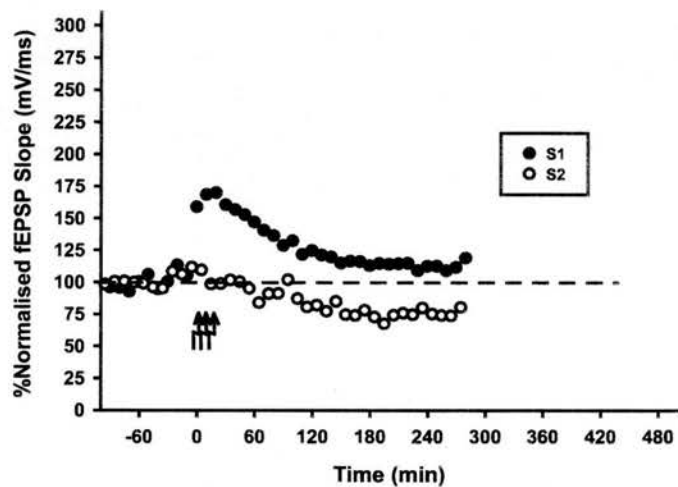
Some individual examples of experiments that had to be abandoned are shown in Figure 3.10. The main problems associated with these recordings fell into two main categories. (1) Experiments in which there was a general decline in the response of both the tetanised and the control pathways (see Figure 3.10 a). (2) Experiments in which the control pathway declined below the 'threshold' of 30% of the pre-tetanus baseline response (see Figure 3.10 b).

The reasons for these problems were difficult to track down. A number of factors (such as those raised in Chapter 2) may have exerted an effect on the long-term viability of the slices in the chamber. Whatever the causes, these examples highlight the importance of having an independent control pathway to correctly interpret either the decay, or persistence, of LTP over long periods. These technical difficulties affected the rate at which certain key experiments were completed, occurring many times during the course of conducting the experiments reported in this thesis.

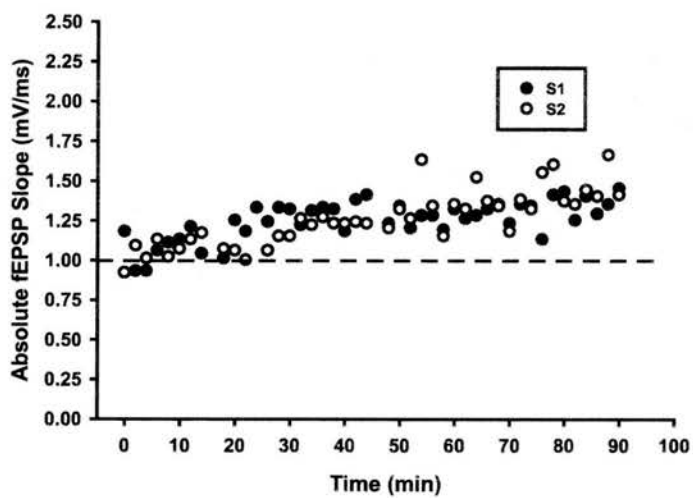
Figure 3.10 Representative examples of ‘failed’ experiments, highlighting the difficulties associated with maintaining hippocampal slices for long-periods in vitro.

- a. An example of an experiment where LTP was induced but the response in both pathways subsequently declined below the ‘threshold’ for inclusion in the overall data set (i.e. > 30 % drift in either pathway). This experiment highlights the necessity in recording from two pathways in order to attribute a decline in LTP to factors intrinsic to the maintenance of LTP and not poor slice health.
- b. An example of an experiment where both pathways were unstable during the pre-tetanus baseline period. Resetting the test pulse stimulation intensity a number of times in order to obtain a 50 % of maximum response did not result in stabilisation of the response. In experiments such as this, if the response of either pathway was found to display a constant drift for > 2 hr, the experiment was terminated and a new slice was chosen.

a.



b.



3.7 Discussion.

In this chapter results have been presented from experiments which have shown that it is possible to record stable responses from hippocampal slices for >6 hr *in vitro*. Long lasting, homosynaptic L-LTP has been successfully induced with a ‘standard’ high-frequency tetanus protocol.

Results were presented from two groups of experiments, indicating that the temperature at which L-LTP experiments are conducted affects the resulting potentiation. L-LTP with recordings at 32 °C resulted in more robust potentiation than that achieved at 28 °C. Many reports have been published whereby L-LTP is induced with a similar multiple high-frequency tetanus protocol as that adopted here (Huang and Kandel, 1994; Nguyen et al., 1994; Abel et al., 1997; Huang et al., 2000; Scharf et al., 2002). In all of these cases, the experiments were conducted at 28 °C, and the authors’ report that L-LTP was induced. One difficulty with this interpretation is that these experiments only lasted 2 – 3 hr post tetanus. It is not entirely clear if difficulties encountered at later time periods. Additionally, in many of these published reports, the experiments were conducted without a control pathway. The present results indicate that the temperature at which L-LTP experiments are conducted is important with respect to the magnitude of L-LTP observed and that the presence of control pathway is critical for monitoring the status of the slice.

Finally, data was presented from individual experiments where technical problems arose during the course of the experiment, thereby limiting their inclusion in the overall data set. These examples highlight that long-term recordings, although possible, are very sensitive to any small fluctuations in the viability of the hippocampal slice *in vitro* during any given experiment.

Chapter 4. Induction of early-phase LTP (E-LTP).

4.1 Introduction.

In Chapter 3, it was established that L-LTP can be induced with multiple trains of high-frequency tetanisation. The experiments reported in this chapter were aimed at addressing the tetanisation requirements for the induction of a shorter lasting, E-LTP.

4.1.1 Dissociating early- from late-LTP.

As has been discussed in the introductory chapter, LTP has been divided into at least two phases, early-LTP (E-LTP) and late-LTP (L-LTP). The division of these different ‘types’ of LTP is defined on the basis of their relative persistence, the dependence/independence on RNA translation, and transcription.

However, there does seem to be some confusion about the exact distinction between E-LTP and L-LTP. “*LTP has an initial phase (I-LTP, also known as short-term potentiation) lasting approximately 30 min that is insensitive to most protein kinase inhibitors, a protein kinase-dependent early phase (E-LTP) that lasts until 3-4 h after induction, and an ensuing late phase (L-LTP) distinguished by a requirement for RNA and protein synthesis at the time of the inducing stimulus*” from Roberson and Sweatt, (1996), p.30436. Huang and Kandel, (1994), found that in the presence of the protein synthesis inhibitor anisomycin (20 μ M), LTP induced by 3 trains started to decay ~30 - 40 minutes after stimulation. In these experiments, LTP was recorded for 3hr post-tetanus. However, if E-LTP is defined as “*a protein kinase-dependent early phase (E-LTP) that lasts until 3-4 h after induction*” should these experiments be taken as evidence that E-LTP involves some degree of protein synthesis? The authors argued that they were dealing with L-LTP as they used the multiple high-frequency tetanus protocol reported to induce an LTP that lasts for 6 – 8 hr (Frey et al., 1988; Frey et al., 1993). They also demonstrated the sensitivity of the potentiation induced by this type of tetanus to inhibitors of PKA (L-LTP requires PKA activity, as discussed in the Chapter 1). They also observed that a shorter lasting E-LTP was induced following a single high-frequency train, this potentiation being unaffected by anisomycin.

4.1.2 The timing of the inhibitory effects of protein synthesis inhibitors.

In their experiments, anisomycin produced an effect quite early in the post-tetanus period (Huang and Kandel, 1994). Experiments by Frey et al., (1988) do not find significant effects of this drug until much later even though a similar dose of the drug was used. In Frey's experiments, L-LTP was induced with a multiple high-frequency tetanus protocol, each train separated by a 10 min interval. Anisomycin (20 μ M) was applied after the third and last train, and was washed out after 3 hours. Relative to a non-tetanised control pathway, significant potentiation of the population spike persisted for the entire duration of the experiment i.e., 8 hr, in the absence of anisomycin. In slices that were incubated with anisomycin, the potentiation was similar for the first 2 hr. After 5 hr, the magnitude of spike potentiation in the drug treated group declined to a level that was significantly lower than control untreated slices. According to Frey et al., (1993), "*The early phase (E-LTP) is initiated typically with a single train of high frequency stimuli, starts immediately after the post-tetanic potentiation induced by the tetanus, and lasts about 1 to 3 hours.*" (p.1661).

4.1.3 Is the persistence of LTP 'set' at the time of tetanisation, or is it influenced by events prior to or after the induction?

The general picture is that the events that lead to L-LTP are initiated immediately at the time of induction, with the triggering of protein synthesis. In contrast, an E-LTP inducing stimulus does not trigger the protein synthesis cascade and only a short-lasting potentiation is produced. The outcome, E-LTP or L-LTP, therefore depends on the tetanus used (i.e., single or multiple trains), with the conceptual dissociation based on the intracellular events triggered at the time of the tetanus. This led to the suggestion that there maybe a specific 'time window' during which L-LTP can be disrupted (Nguyen et al., 1994), and that the extent of persistence is set at the time of LTP induction. However, there are now grounds to suspect that both suggestions are misleading.

The importance of making a clear distinction between E-LTP and L-LTP, in regard to the issues raised above, is highlighted by the synaptic tag experiments of Frey and Morris, (1997, 1998a). These experiments revealed that the duration of LTP is determined by conditions that are active prior, during and after the moment of tetanisation. Specifically, the persistence of LTP is subject to heterosynaptic influences both prior and subsequent to the events (i.e., tetanic stimulation) which are responsible for triggering some of the cascades involved in L-

LTP. The synaptic tagging experiments indicate that E-LTP (induced by a single high-frequency tetanus) can be ‘converted’ into L-LTP if multiple high-frequency tetanisation is applied to a second independent input to the same group of CA1 neurons within a critical time window. These findings point to a very different perspective concerning the differentiation between E-LTP and L-LTP. The distinction remains based on the duration of each type of potentiation, but the underlying mechanisms are different. Persistence need not be set only at the moment of tetanisation, and therefore the notion of there being only a post-induction time-window is misleading.

E-LTP may be a first step in the process that can, depending on the ‘circumstances’, become a more enduring L-LTP. These ‘circumstances’ include the prior state of the neurons that are activated by stimulation normally inducing E-LTP. If prior neuronal activity led to protein synthesis, then synapses stimulated at a level that induces only E-LTP may also display L-LTP. So rather than has been suggested by Lanahan and Worley, (1998) “*macromolecular synthesis is required only during a brief time window immediately following a conditioning stimulus*” (p.37), the persistence of LTP is more likely to result from the interaction of two dissociable events. The first involves local activation of specific synapses in a way that tags them for a certain period. The second is the diffuse signalling to the cell body to synthesise proteins that upon distribution to the dendrites are captured by tags involved in the long-term changes defined as L-LTP.

As a first step towards asking some of these important questions concerning the interaction between ‘local’ synaptic tags, and the ‘diffuse’ distribution of plasticity-related proteins, it was important first to establish a method for the induction of E-LTP. This should be a clear and statistically significant potentiation lasting at least one hour followed by a return to a baseline level that does not differ from a control pathway or the pretetanus baseline level. These experiments are reported in the following sections.

4.2 Methods

4.2.1 Preparation of slices, and recording set-up.

Similar procedures for the preparation and incubation of slices were used as described Chapters 2 and 3.

4.2.2 Tetanus protocol.

For the induction of E-LTP, the tetanus protocol consisted of a single high-frequency tetanic train of 21 pulses at 100Hz (biphasic pulses with 200 μ sec per half-wave i.e. twice the duration of test stimulation. Experiments in which the drift in the control non-tetanus pathway was >30% in either direction, over 6 hr post-tetanus period were excluded from analysis. Recordings were made for at least 6 hr post-tetanus.

4.3 Results.

4.3.1 Assessment of maximal response (input/output measurement).

The averaged response over a range of stimulation intensities from 10 – 90 μ A increased from 0.40 to 4.17 mV/ms for S1, and from 0.24 to 3.87 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

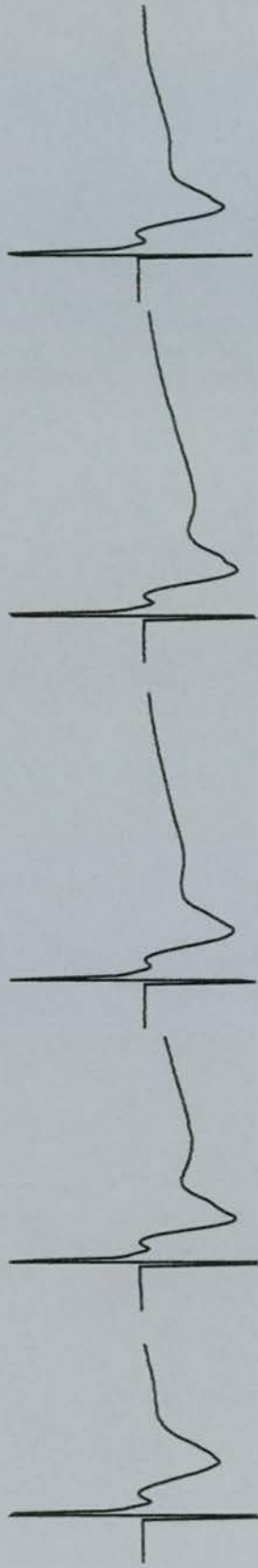
4.3.2 Stable baseline responses and analysis of post-tetanic potentiation (PTP): 21 pulse tetanus experiments.

Representative field EPSPs are shown from an individual experiment in Figure 4.1. Statistical comparison with a one-sample t-test where the normalised response from each pathway was compared to the baseline level of 100 %, showed that there was no PTP in the control pathway after delivery of the first of the three tetani (fEPSP Slope = 101.8 % \pm 1.9 s.e.m., $t = 0.92$, $p > 0.39$) compared to the baseline level. The tetanised pathway showed robust PTP (fEPSP Slope = 157.8 % \pm 10.2 s.e.m., $t = 5.69$, $p < 0.01$).

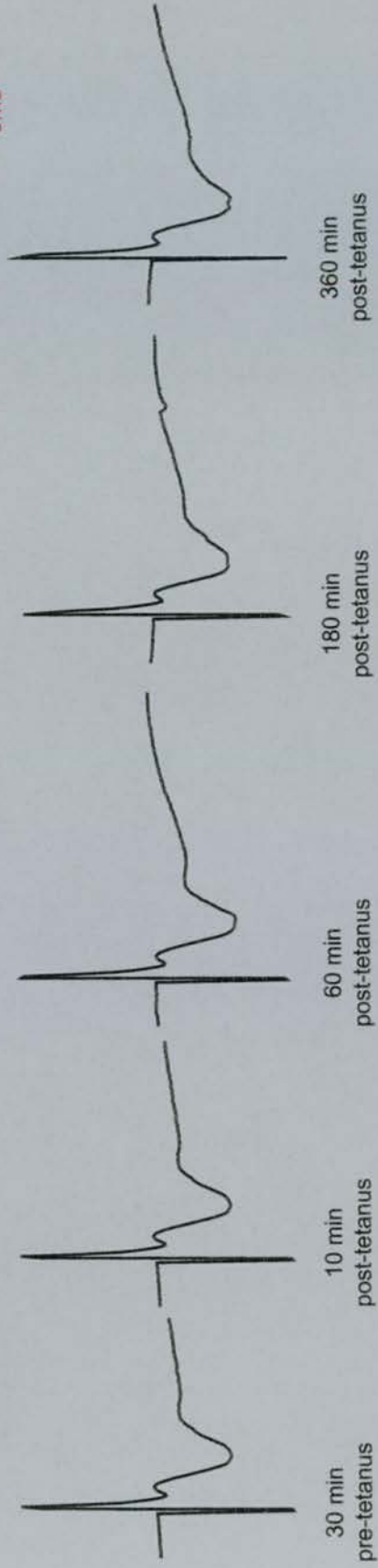
Figure 4.1 Representative example of fEPSP waveforms from an individual experiment with 21 pulses at 100 Hz.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 4.2 a.

S1 - Tetanised pathway



S2 - Non-tetanised pathway



4.3.3 Experiments reveal the unexpected induction of L-LTP with a single high-frequency tetanus.

An individual example of one of the experiments, which form part of the grouped data presented later, is shown in Figure 4.2 a. A tetanus of 21 pulses at 100Hz was delivered to the S1 pathway. The response was 154.56 % of the pre-tetanus baseline level at 10min post-tetanus. The level of potentiation remained at a level of 143.53 % at 360min post-tetanus. The control pathway (S2) showed no change at any of these time points.

Grouped data (n=7) is shown in Figure 4.2 b. A repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors was conducted. There was a significant difference between the two pathways [$F(1, 6) = 61.1$; $p < 0.005$], indicating that the tetanus produced a robust LTP that was non-decremental. The level of potentiation was 141.3 % at 10 min post-tetanus, and remained elevated at 128.9 % 6 hr post-tetanus. No significant pathway x time interaction was found [$F(3, 18) = 2.1$; $p > 0.05$]. As with multiple tetani, the level of potentiation declined by a small amount in the initial stages but stabilised thereafter [$F(3, 18) = 10.9$; $p < 0.005$]. There was no significant change in the control pathway [$F(3, 18) = 0.5$; $p > 0.05$].

4.3.4 Summary of experiments.

In summary, the tetanised pathway (S1) showed robust potentiation initially after a single high frequency tetanus of 21 pulses. This LTP subsequently declined but was still significantly greater compared to the control pathway (S2) at 6 hr. The non-tetanised control pathway (S2) remained very stable for the entire post-tetanus period. The averaged values \pm s.e.m. are presented in Table 4.3. Statistical comparisons between the tetanised and the control pathways at various time points during the experiment (Figure 4.2 c) showed significant differences at all times. The magnitude of the potentiation induced with 21 pulses was significantly less than that induced with three trains of high frequency stimulation. At 360 min post-tetanus, fEPSP Slope = 171.4 % for three trains and 128.9 % for 21 pulses. There was a significant difference between these experiments [$F(1, 17) = 19.5$; $p < 0.005$] indicating that more LTP was induced with three trains compared to a single train of 21 pulses.

Figure 4.2 The unexpected induction of L-LTP with a single high frequency tetanus.

- a. An individual experiment showing a representative example of the potentiation induced with a single tetanus of 21 pulses at 100 Hz (the waveforms in Figure 4.2 are from this experiment).
- b. Grouped data for experiments in which LTP was induced with a single tetanus of 21 pulses ($n = 7$). For all experiments, the electrodes were positioned within the stratum radiatum. Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. Baseline (100 %) was taken as the mean of the values in consecutive 10 min periods (i.e. 5 successive evoked responses), and 60 min of pre-tetanus baseline response was recorded before a single tetanus of 21 pulses at 100 Hz was delivered to S1 (indicated by arrow). Error bars indicate \pm s.e.m. Symbols: S1 tetanised pathway ●. S2 non-tetanised pathway ○.
- c. Statistical comparisons were made between the tetanised pathway (S1) and the non-tetanised control pathway (S2) after the tetanus at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at each time point. *** indicates a statistically significant difference with $p < 0.005$, ** with $p < 0.01$ (paired students t – test, with Bonferroni correction for multiple comparisons). Significant differences were observed at all times.

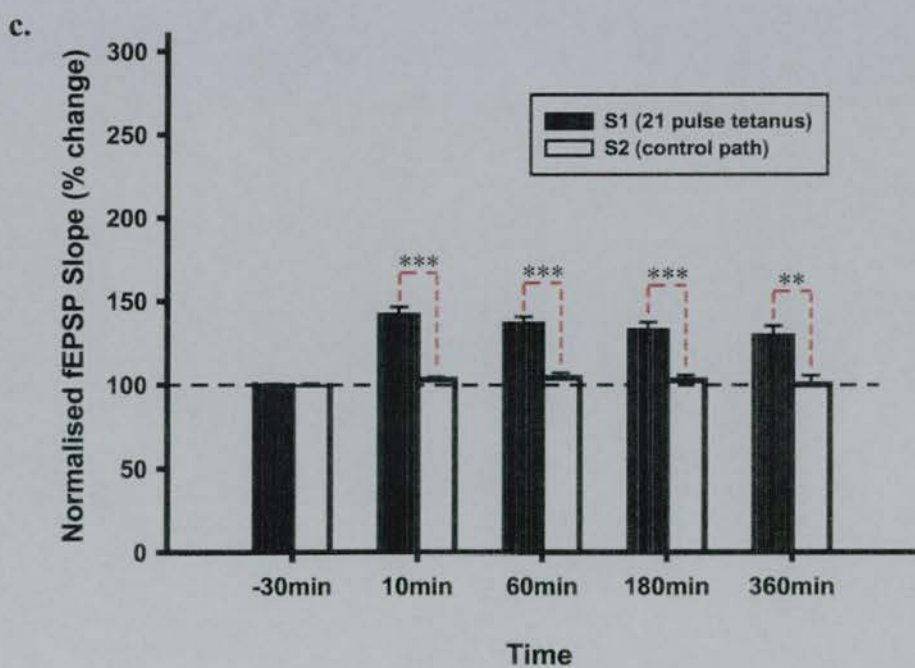
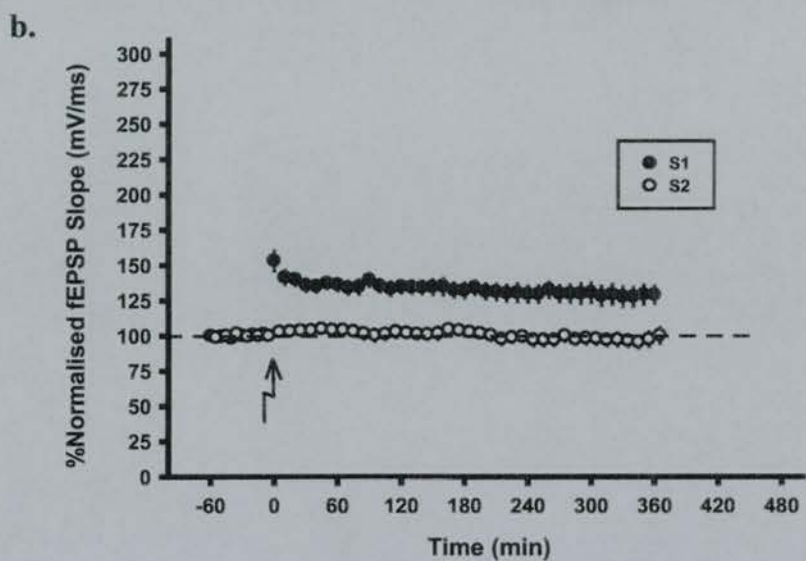
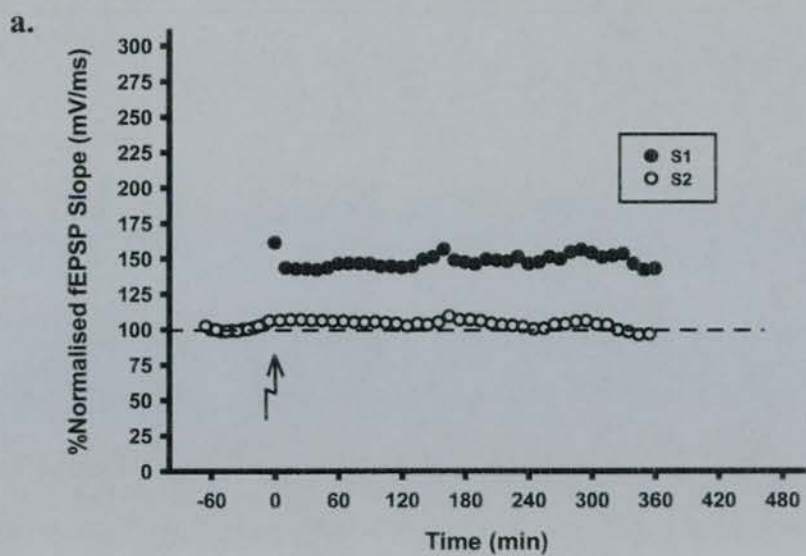


Table 4.3 Summary of statistical comparisons for 21 pulse tetanus experiments.

Time (min)	S1 pathway normalised EPSP slope	S2 pathway normalised EPSP slope	t value	
-30	100.14 ± 0.52	99.54 ± 1.34	0.66	p >0.5
10	141.33 ± 5.20	103.20 ± 1.65	7.53	p <0.005
60	136.26 ± 4.44	104.13 ± 3.04	10.63	p <0.005
180	132.08 ± 5.35	102.61 ± 3.08	9.49	p <0.005
360	128.89 ± 6.35	100.11 ± 5.54	4.10	p <0.01

Table 4.3: Paired samples t – test comparisons of S1 and S2 pathways, at a number of times before and after the tetanus. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

4.4.1 Reducing the number of pulses in a tetanus produces E-LTP.

Frey and Morris, (1997, 1998a) reported that a single tetanus of 21 pulses at 100Hz induced a short-lasting E-LTP in the CA1. Similar stimulation parameters were used in these published and my own experiments (i.e., 200 μ sec pulses, biphasic square wave pulses). It was therefore somewhat surprising to find that L-LTP (albeit of a smaller magnitude than that induced with three trains of 100 pulses at 100Hz), was produced with a single 100Hz tetanus of 21 pulses. However, subtle differences in chamber design, the orientation of 'bath' electrode, or other factors may result in 21 pulses having different physiological effects in one lab to that in another. Frey and Morris, (1997) reported that a single train of 11 pulses (100Hz, biphasic pulses 100 μ sec per half wave), resulted in a short-lasting LTP that persisted for 1 – 2 hr post-tetanus. Experiments were therefore conducted in which one pathway received a single tetanus of 11 pulses at 100Hz.

4.4.2 Assessment of maximal response (input/output measurement).

The averaged response over a range of stimulation intensities from 10 – 90 μ A increased from 0.64 to 3.52 mV/ms for S1, and from 0.11 to 3.76 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

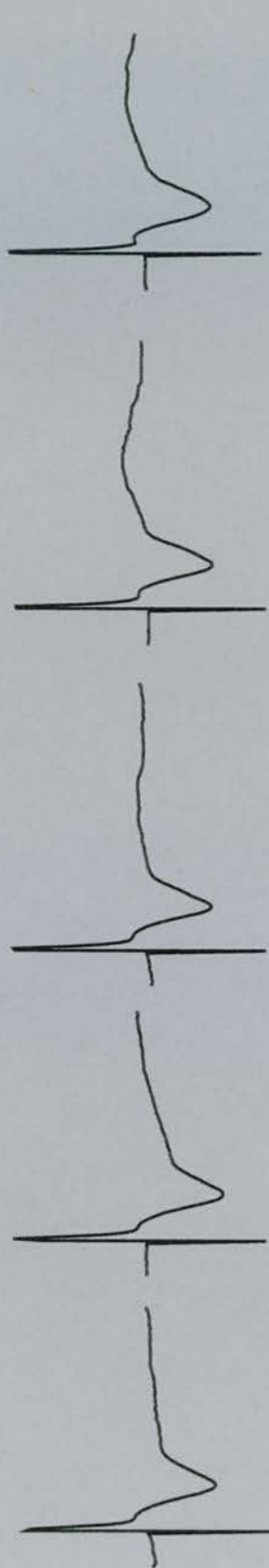
4.4.3. Stable baseline responses, and analysis of post-tetanic potentiation (PTP).

Representative field EPSPs are shown from an individual experiment in Figure 4.4. Statistical comparison with a one-sample t-test where the normalised response from each pathway was compared to the baseline level of 100 %, showed that there was no PTP in the control pathway after delivery of the first of the three tetani (fEPSP Slope = $102.7 \% \pm 3.1$ s.e.m., $t = 0.87$, $p > 0.41$) compared to the baseline level. The tetanised pathway showed robust PTP (fEPSP Slope = $156.0 \% \pm 5.4$ s.e.m., $t = 5.69$, $p < 0.005$).

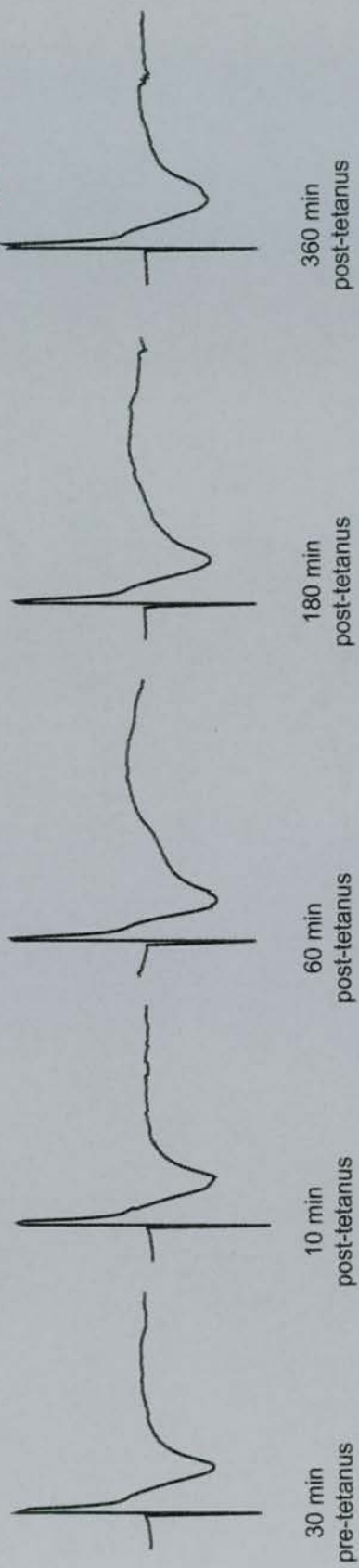
Figure 4.4 Representative example of fEPSP waveforms from an individual experiment with 11 pulses at 100 Hz.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 4.5 a.

S1 - Tetanised pathway



S2 - Non-tetaniised pathway



4.4.4 E-LTP is induced with a single tetanus of 11 pulses.

An individual example of one of the experiments, which form part of the grouped data presented later, is shown in Figure 4.5 a. The tetanus (11 pulses at 100Hz) was delivered to the S1 pathway, 10min post-tetanus the response was 144.88 % of the pre-tetanus baseline level. The level of potentiation had completely declined to a stable level of 98.05 % at 360min post-tetanus. The control pathway (S2) showed no change at any of these time points.

The results of experiments (n=8) are shown in Figure 4.5 b. A repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors was conducted. There was a significant difference between the two pathways [$F(1, 7) = 24.1$; $p < 0.01$], indicating that significant potentiation resulted from this single tetanus. Critically, a significant pathway x time interaction was found [$F(3, 21) = 16.6$; $p < 0.005$]. Subsequent analysis revealed that this interaction i.e., the decline in response across time, was due to a significant decay in level of potentiation across time indicating that E-LTP was induced [$F(3, 21) = 23.9$; $p < 0.005$], and not because of any change in the control pathway across these intervals [$F(3, 21) = 0.6$; $p > 0.05$].

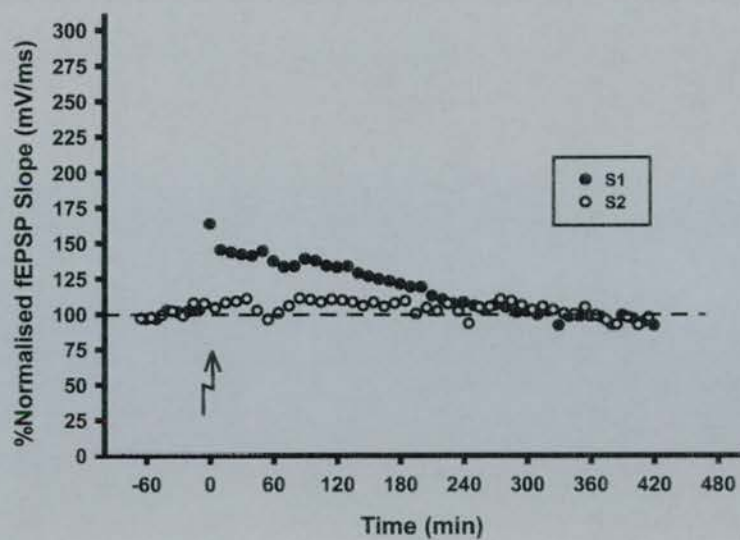
4.4.5. Summary of experiments.

In summary, the tetanised pathway (S1) showed LTP only for the initial 2 hr after a single high frequency tetanus of 11 pulses. This potentiation declined and was not significantly greater compared to the control pathway at 180 min post-tetanus. The non-tetanised control pathway (S2) remained very stable for the entire post-tetanus period. The averaged values \pm s.e.m. are presented in Table 4.6. Statistical comparisons between the tetanised and the control pathways at various time points during the experiment, shown in Figure 4.5 c., revealed significant differences only at the later time points, indicating that this type of tetanus induced a shorter lasting E-LTP.

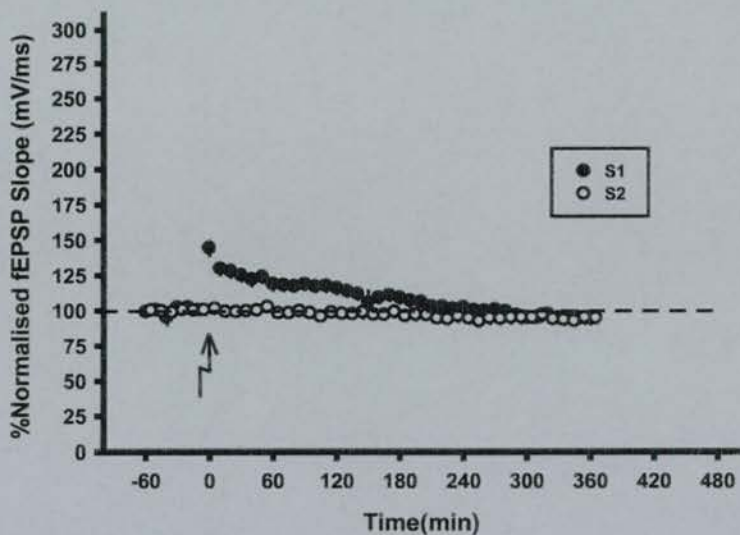
Figure 4.5 The induction of E-LTP with a single tetanus of 11 pulses at 100 Hz.

- a. An individual experiment showing a representative example of the potentiation induced with a single tetanus of 11 pulses at 100 Hz.
- b. Grouped data for all experiments in which LTP was induced with a single high frequency tetanus ($n = 8$). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. Baseline (100 %) was taken as the mean of the values in consecutive 10 min periods (i.e. 5 successive evoked responses), and 60 min of pre-tetanus baseline response was recorded before a single tetanus of 11 pulses at 100 Hz was delivered to S1 (indicated by arrow). Error bars indicate \pm s.e.m. Symbols: S1 tetanised pathway ●. S2 non-tetanised pathway ○.
- c. Statistical comparisons were made between the tetanised pathway (S1) and the non-tetanised control pathway (S2) after the tetanus at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at each time point. *** indicates a statistically significant difference with $p < 0.005$, ** with $p < 0.01$ (paired students t – test, with Bonferroni correction for multiple comparisons). Significant differences were observed at 120 min but not 180min post-tetanus.

a.



b.



c.

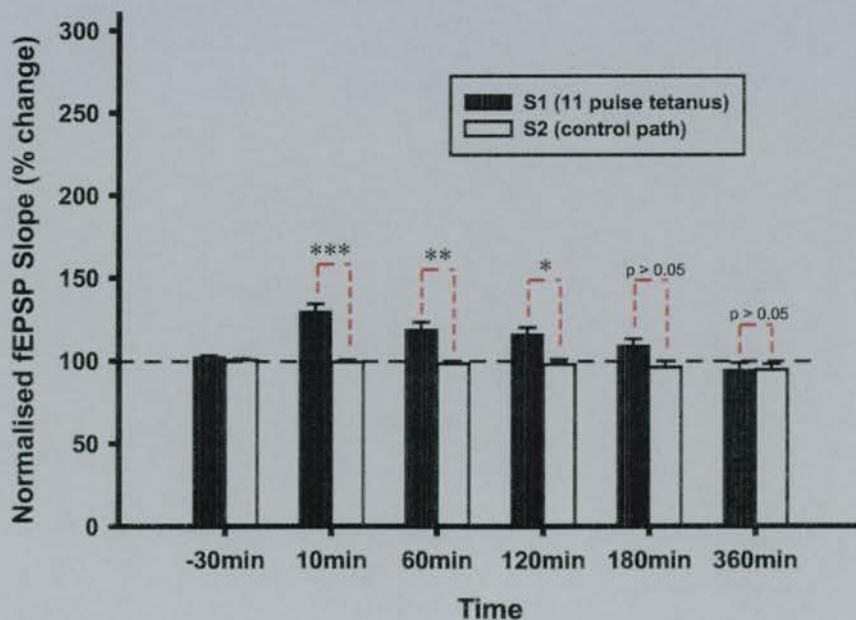


Table 4.6 Summary of statistical comparisons for experiments with an 11 pulse tetanus at 100 Hz.

Time (min)	S1 pathway normalised EPSP slope	S2 pathway normalised EPSP slope	t value	
-30	102.30 ± 0.98	100.97 ± 0.80	1.05	p > 0.3
10	129.76 ± 4.45	99.23 ± 1.36	7.37	p < 0.005
60	119.37 ± 4.15	98.16 ± 1.63	5.23	p < 0.005
120	115.87 ± 4.03	97.73 ± 3.09	5.39	p < 0.005
180	109.14 ± 3.87	96.09 ± 3.64	3.10	p > 0.05
360	95.08 ± 3.77	95.23 ± 3.76	< 1	p > 0.9

Table 4.6: Paired samples t – test comparisons of S1 and S2 pathways, at a number of times before and after the tetanus. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

4.5 Discussion.

The experiments reported in this chapter reveal that with a specific LTP induction protocol of 11 pulses delivered at 100 Hz, induces E-LTP that persists for approximately 1 – 2 hr post-tetanus. Increasing the number of pulses in the train to 21 results in a stable long-lasting L-LTP. This is smaller in magnitude to that induced by multiple trains of 100 pulses.

It was important to establish stimulation parameters by which a short lasting form of potentiation could be induced. As the experiments reported in this chapter highlight, single trains of high frequency stimulation may be sufficient to induce L-LTP. As was mentioned in the introductory section of this chapter, a distinction between E-LTP and L-LTP is sometimes made in terms of the tetanus used to induce either early or late-LTP (i.e., single or multiple high-frequency trains). This has led to the suggestion that there is a specific ‘time window’ during which L-LTP can be disrupted (Nguyen et al., 1994), and that the time is set at the time of LTP induction.

Protein synthesis inhibitors can be present either before, during, or very shortly after a high frequency tetanus to produce a block of L-LTP. This may result from the requirements of the experimental set up, i.e., in order to allow the drug to reach the slices at a high enough concentration to penetrate the cells of the slice and to produce a block. Protein synthesis inhibitors require several minutes to penetrate the cell, bind to ribosomes, and inhibit protein synthesis (Grollman, 1968). Deadwyler et al., (1987), found a reduced but incomplete LTP blockade in slices when protein synthesis inhibition was 79 % after a 15 min pre-incubation period, relative to a 30 min pre-incubation period when the level of inhibition had risen to 85 %.

A high frequency tetanus can be used to upregulate protein synthesis immediately at the time of LTP induction, although this may be an experimentally convenient method than reflective of what happens *in vivo*. The levels of protein synthesis are likely to be constantly changing in a dynamic way, rather than being phase-locked to any one signal. Rather than a ‘time-window’ arising from events triggered at a discrete moment in time, the window may arise from an interaction between a local synaptic modification (that may be triggered by the induction of E-LTP) and a more diffuse signal to the cell body which causes the synthesis and distribution of plasticity proteins throughout the cell. This alternative perspective has been put

forward by Frey and Morris, (1998b). The basic idea is that a tetanus that 'normally' would result in E-LTP may activate a local synaptic tag that remains active for a certain period of time. If something like high frequency stimulation occurs to upregulate protein synthesis within this time, these proteins may be distributed throughout the cell but only utilised at synapses which were previously (or subsequent to the high frequency tetanus) active, and therefore 'tagged'. On this view, any time-window results from an interaction between the decay kinetics of the tag and the availability of proteins.

In order to address the issue of synaptic tagging and capture, it is necessary to establish that different patterns of tetanic stimulation induce LTP that varies in terms of its duration after the tetanus. The findings of Chapter 3, reveal that multiple tetanic trains induce robust L-LTP, whereas a single train of 11 pulses results in E-LTP that persists for only 1 – 2 hr.

Chapter 5. Synaptic tagging, the variable persistence of LTP.

5.1 Introduction.

In chapter 4, it was established that reducing the number of pulses within a single high frequency train produced a decremental form of potentiation (E-LTP). The experiments in this chapter tested whether or not this decremental form of LTP could be ‘transformed’ into L-LTP by either prior, or subsequent, tetanisation to a second independent input to the same population of CA1 neurons.

5.1.1. The Synaptic Tag hypothesis.

In Chapter 1, a number of properties of LTP were discussed. NMDA receptor dependent LTP is input specific, as only activated synapses undergo the long-lasting changes in synaptic efficacy that are associated with LTP. It was also noted that, for L-LTP to remain persistent for long periods, both protein translation and transcription are required. Each CA1 pyramidal neuron possesses dendrites that may bear over 10,000 synapses, each capable of being modified independently of one another. A problem that then arises is to understand how potentiated synapses of a single neuron become selectively modified during L-LTP, if this process is dependent upon events, such as transcription and translation that occur primarily in the cell body of the neuron (Wenzel et al., 1993). If a signal must be sent to the nucleus to trigger transcription and translation of proteins, then we are left with a ‘navigational’ problem of getting the proteins to the exact synaptic site where they are required to stabilise the potentiated state of an individual synapse. The synaptic tag hypothesis suggests a potential answer to this dilemma.

The synaptic tag experiments of Frey and Morris, (1997) revealed that potentiated synapses become tagged in a protein synthesis independent manner that allows them to interact with somatically synthesised “*plasticity proteins*” during L-LTP. These experiments involved stimulation of two independent input pathways to the same CA1 neuronal population. In the first series of experiments, ‘strong’ tetanic stimulation (consisting of three 100 Hz trains separated by 10 min intervals) was delivered to one pathway (S1), and subsequent to this, the protein synthesis inhibitor anisomycin was added to the bath 35 min after the tetanus, a time when it is ineffective in blocking the potentiation induced in S1, (Otani et al., 1989). A

second 'strong' tetanus was then delivered (1 hr after the S1 tetanus) to an independent, but overlapping, input pathway (S2) to the same population of CA1 neurons, but this time anisomycin was present during this tetanus. L-LTP that would normally be blocked when induced in the presence of anisomycin, was induced in both the S1 and S2 pathways. The authors suggest that induction of LTP in the presence of anisomycin (i.e., the S2 pathway) resulted in the setting of a synaptic tag that was capable of utilising proteins that were synthesised in response to the prior 'strong' tetanus to the S1 pathway, therefore L-LTP was induced in S2.

Subsequent experiments showed that the synaptic tag is only transiently set at activated synapses, and that there is a 'time-window' in which the proteins distributed throughout the cell can interact with the tag. If one pathway (S1) was tetanised in the presence of anisomycin, before a second pathway (S2) was tetanised (the drug was washed out 2.5 hr before the S2 tetanus), L-LTP was induced on S2. The LTP induced on S1 was decremental and returned to basal levels after approximately 4-5 hr.

5.1.2 Heterosynaptic modulation of LTP: 'Strong before weak' and 'Weak before strong' experiments.

These experiments indicate that L-LTP involves two dissociable events; first L-LTP is dependent upon protein synthesis; second it is also critically determined by another event, the transient setting of a synaptic tag during the early stages of LTP. The input-specificity of L-LTP may be achieved through an interaction between the 'local' protein synthesis independent synaptic tag, and the 'diffuse' synthesis of proteins that is believed to occur primarily in the cell body. Frey and Morris, (1997) conducted experiments to determine the events underlying the setting of synaptic tags. In one series L-LTP was induced by a 'weak' tetanus that ordinarily only induces E-LTP, when this was preceded by the induction of L-LTP on an independent pathway.

If the setting of synaptic tags can be dissociated from the triggering of protein synthesis, the order of these events should be unimportant. Frey and Morris, (1998a) conducted experiments where the order of 'strong' and 'weak' stimulation was reversed, so-called 'Weak before Strong' experiments. In the critical series, when a single, 'weak' tetanus in S1 was followed by a 'strong' tetanus in S2, early-LTP could be transformed into late-LTP if the 'strong' tetanus was applied within 60 min of the 'weak' tetanisation of S1. The heterosynaptic

stabilising effect of the ‘strong’ tetanisation was much less at an interval of 2 hr, and absent at 4 hr. The ‘weak before strong’ experiments suggest that ‘tags’ decay over 1–2 hr. Tag setting may therefore involve transient processes such as protein phosphorylation. It also suggests that proteins upregulated many hours after the induction of LTP are unlikely candidate synaptic tags, although the precise identity of the tag (or tags) is not known at present (candidate synaptic tags were discussed in Chapter 1, and have been recently reviewed by Martin and Kosik, 2002).

5.1.3 The present experiments.

The experiments that were conducted in this chapter focussed on replicating, in Edinburgh, the major aspects of the experiments of Frey and Morris, (1997, 1998a). First, is the persistence of LTP influenced by heterosynaptic inputs to the same CA1 neurons? This constituted a replication of the ‘Strong before weak’ and ‘Weak before strong’ experiments. Second, does this rescue effect arise due to the triggering of translation by a repeated high frequency tetanus? Specifically, do translational inhibitors block L-LTP?

The first of these two questions asks whether the ‘Weak before Strong’ and ‘Strong before Weak’ effect is reproducible. The reason why this is important to consider emerges upon closer inspection of the data from the experiments of Frey and Morris, (1998a). In ‘control’ experiments L-LTP was induced with three trains of high frequency stimulation (‘strong’ stimulation). This induced a persistent potentiation of the stimulated S1 pathway. However, over the course of the post-tetanus period, the response of the S2 non-tetanised control pathway was found to increase, reaching a level of ~ 15 – 20 % 7 hr post-tetanus. This is the baseline against which a ‘rescued’ L-LTP should really be assessed. A second series of experiments were conducted where E-LTP was induced with a single tetanus of 21 pulses at 100 Hz (‘weak’ stimulation). This induced potentiation that was elevated for the first 2 – 3 hr relative to the control S2 pathway. Again however, there were changes in the S2 non-tetanised pathway. This time the response of the S2 pathway declined during the post-tetanus period by ~ 15 – 20 % after 7 hr. The E-LTP on S1 declines to the pre-tetanus baseline, but it may have been elevated relative to the S2 pathway. Had S2 been ‘subtracted’, a persistent L-LTP may have been present statistically – as I found in Chapter 4 when stimulating with a 21 pulse tetanus.

These findings may not be as surprising if the following two points are considered. First, if 'strong' stimulation of S1 always results in a 'heterosynaptic' increase in a second independent pathway, then it may not be that surprising that subsequent 'weak' stimulation of S2 results in a persistent L-LTP on this pathway. Second, with a 'weak' tetanus alone, the S2 control pathway was always seen to decline in the post-tetanus period. If this decay in the S2 pathway was not present, the response of the S1 tetanus pathway may have remained elevated at ~ 110 – 115 % of the pre-tetanus baseline level even after 7 hr. If this was also the case when 'weak' stimulation occurred prior to 'strong' stimulation then once again it may not be surprising to have recorded L-LTP on this pathway. The magnitude of L-LTP in the 'weak' stimulated pathway when followed by 'strong' stimulation was less than that of the 'strong' tetanus pathway – at a level of approximately 130 % after 7 hr.

It is interesting that the experiments reported in Chapter 4 revealed that a single tetanus of 21 pulses resulted in persistent L-LTP, albeit at a reduced magnitude (128.9 % vs. 173.5 % for 'weak' alone and 'strong' alone respectively) to that induced with multiple high frequency trains. In my experiments, both 'weak' and 'strong' LTP occurred against a background of a stable control pathway. Therefore, replication of the 'weak before strong' experiments on this background of baseline stability would clarify these alternative interpretations discussed above. Ideally, such an experiment would incorporate a third non-tetanus pathway to serve as a within-slice control. While its desirability was discussed, time constraints precluded the extensive preparatory work that would have been necessary to incorporate such a control.

The interpretation of the ability of 'strong' stimulation to transform a normally decremental form of potentiation, or E-LTP, into non-decremental L-LTP is based on the idea that plasticity-proteins triggered by a 'strong' tetanus can interact with synaptic tags set at 'weak' activated synapses. Therefore, experiments were conducted with protein synthesis inhibitors present during 'strong' stimulation. Control experiments were also conducted to confirm that the application of protein synthesis inhibitors to hippocampal slices was associated with a block of translation. This was assessed by measuring the incorporation of [³⁵S]-methionine in hippocampal slices.

5.2 Methods.

5.2.1 Preparation of slices, and recording set-up.

Similar procedures for the preparation and incubation of slices were used as described Chapters 2 and 3.

5.2.2 Tetanus protocol.

In the experiments reported in this chapter, two different tetanus protocols were used. Either 'strong' or 'weak' high frequency stimulation was used to induce LTP, and this nomenclature is used to differentiate between these protocols throughout this chapter. 'Strong' stimulation consisted of three high-frequency tetanic trains at 100 Hz in a 1 s burst, with an inter-train interval of 10 min (biphasic pulses with 200 μ s per half-wave i.e. twice the duration of test stimulation), i.e., a protocol which was shown to induce robust L-LTP (Chapter 3). 'Weak' stimulation consisting of a single train of 11 pulses at 100Hz (biphasic pulses 200 μ sec per half wave) i.e., a protocol which was shown to induce E-LTP (Chapter 4).

5.2.3 Preparation of drugs.

In order to determine whether protein synthesis inhibitors affected L-LTP, two drugs previously reported to block L-LTP were tested. The translation inhibitor anisomycin (either 25 or 50 μ M), was prepared as a stock solution and stored at -20°C . In preparation of stocks of the drug, the powdered drug was dissolved in aCSF with a small volume of 0.1M HCl, in order to get the drug into a soluble form. The pH was then confirmed to be similar to that of the aCSF, i.e., 7.3 – 7.5. Emetine (100 μ M), was also prepared as a stock solution (1mM) and stored at -20°C . In preparation of stocks of drug, the powdered drug was dissolved in the aCSF alone and gentle sonication was required to get the drug into solution. The pH was then confirmed to be similar to that of the aCSF, i.e., 7.3 – 7.5. Both drugs were bath applied to slices by switching the tubing between reservoirs. The perfusion flow rate was maintained at 150 μ l/min and drug application times were corrected for transit time from the reservoirs to the slice (15 min). The drug was applied 30 min prior to tetanisation, and remained on the slices until 60 min after the tetanus.

5.2.4 Measurement of protein translation with [³⁵S]-methionine.

I am indebted to Dr. Kris Dickson of the Division of Neuroscience, University of Edinburgh, who kindly conducted the protein translation assay with [³⁵S]-methionine.

In order to determine whether protein translation was blocked in the presence of the emetine, experiments were conducted to measure the incorporation of [³⁵S]-methionine in hippocampal slices. Slices (400 µm) were prepared according to identical procedures as used in electrophysiological experiments. The general protocol for these experiments was as follows.

Slices were removed from the recording chamber and incubated in 250 µl aCSF with 2.5 µl *in vivo* cell-labelling grade [³⁵S]-methionine (at 10 µCi/µl = 0.37 MBq/µl) at 32°C for 1 hour. Following this, the free label was washed off by placing the slice in a petri dish filled with an excess of aCSF; this was repeated two more times. The slice was then removed to an eppendorf tube and quickly frozen on dry ice. The slice was homogenised in 30 µl DOC buffer using a plastic pestle, and the homogenate was stored on ice for 10 minutes. The homogenate was then spun down for 10 min at 4°C and 25 µl of supernatant was collected, avoiding any precipitate at the bottom of the tube. Protein concentration was quantified by Bradford assay. Between 25 - 40 µg protein was run out (equal amount per lane) on a 10% protein gel, at 100 V, for 1.5 hr. The gel was then fixed for 10 minutes in 10% HOAc/10%MeOH and following this was enhanced for 1 hr in En3Hance solution. The gel was then washed for 30 min in dH₂O and then dried for at least 1 hour at 80°C. before being placed on film or a phosphor-screen overnight.

5.3 Results.

The first set of experiments were aimed at determining whether or not ‘strong’ stimulation to one input pathway (S1) had any effect on the potentiation induced by ‘weak’ stimulation of a second independent input pathway (S2). The interval between the ‘strong’ and ‘weak’ tetani was 50 min.

5.3.1 Assessment of maximal response (input/output measurement): ‘Strong before weak’ experiments.

The averaged response over a range of stimulation intensities from 10 – 90 μ A increased from 0.57 to 2.92 mV/ms for S1, and from 0.69 to 2.83 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

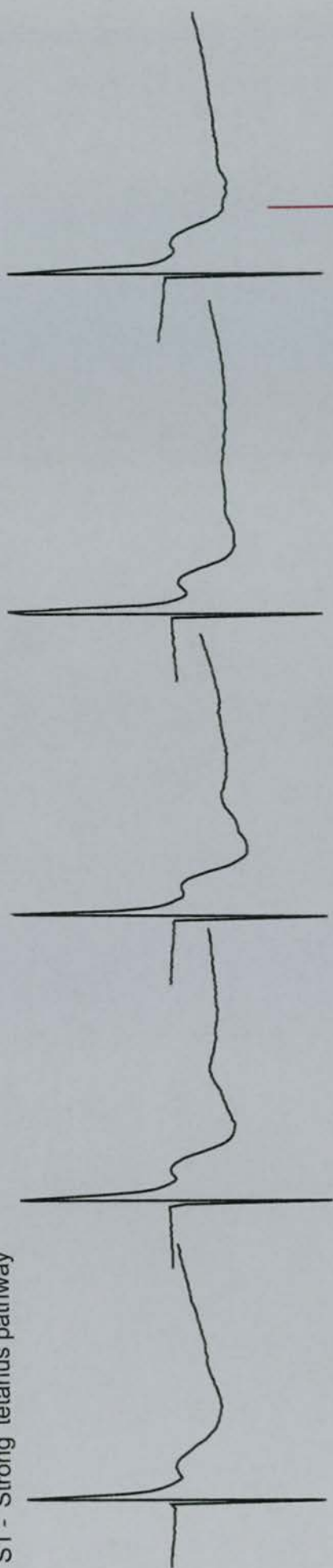
5.3.2 ‘Strong before weak’ experiments: Stable baseline responses, and analysis of post-tetanic potentiation (PTP).

Representative field EPSPs are shown from an individual experiment where repeated ‘strong’ stimulation of one input pathway (S1) preceded (50 min interval) a single ‘weak’ stimulation of a second independent input pathway (S2) to the same group of CA1 cells Figure 5.1. There was no PTP in the S2 pathway after delivery of the first of the three tetani to the S1 ‘strong’ pathway (fEPSP Slope = 106.67 % \pm 5.75 s.e.m., $t = 1.16$, $p > 0.3$) compared to the baseline level. The S1 pathway showed robust PTP (fEPSP Slope = 187.93 % \pm 6.03 s.e.m., $t = 14.59$, $p < 0.005$). ‘Weak’ tetanisation of S2 resulted in significant PTP in this pathway after the tetanus (fEPSP Slope = 151.05 % \pm 5.73 s.e.m., $t = 8.90$, $p < 0.005$).

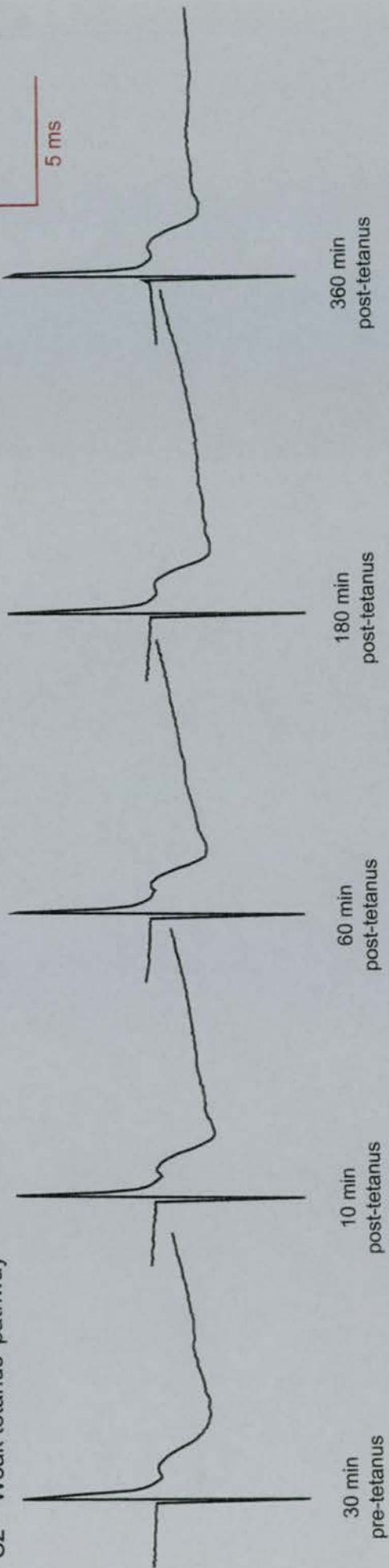
Figure 5.1 Representative fEPSPs waveforms from an individual ‘Strong before Weak’ experiment.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 5.2 a.

S1 - 'Strong' tetanus pathway



S2 - 'Weak tetanus' pathway



5.3.3. Rescue of decremental LTP by a prior ‘strong’ tetanus.

An individual example of an experiment where the S1 pathway was given ‘strong’ tetanic stimulation 50 min before S2 received ‘weak’ stimulation is shown in Figure 5.2 a. Stable baseline responses were recorded for 1 hr before S1 was tetanised (‘strong’ stimulation). This ‘strong’ tetanus induced robust LTP, with the level of potentiation being 178.26 % 10 min post-tetanus, and this remained elevated at 141.80 %, 420 min post-tetanus. The ‘weak’ tetanus resulted in an initial potentiation of 140.25 %, 10 min after this tetanus (i.e., 60 min after the ‘strong’ tetanus), rather than being decremental, this remained elevated for the entire post-tetanus period reaching a level of 126.76 % at 420 min.

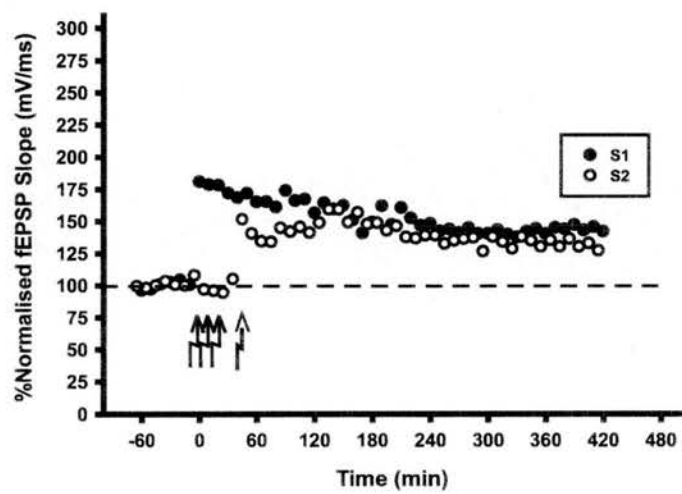
The results of the ‘Strong’ before ‘Weak’ experiments (n=6) are shown in Figure 5.2 b. In order to determine whether or not ‘Strong’ tetanisation had any effect on the duration of the potentiation induced with a subsequent ‘Weak’ tetanus, comparisons were made between these ‘Strong before Weak’ series of experiments, and experiments in which ‘Weak’ stimulation alone was delivered to just one pathway (these ‘weak’ alone experiments, presented in Chapter 4, were run in conjunction with the ‘Strong before weak’ experiments on alternate days). A repeated measures ANOVA with time (10, 60, 180, 360 min) after the ‘weak’ tetanus as a within-subjects factor and experimental condition (‘Weak’ tetanus alone or ‘Strong before Weak’) as a between-subjects factor was conducted. There was a significant difference between the experiments [$F(1, 12) = 13.78$; $p < 0.01$] indicating that a ‘Strong’ tetanus delivered 50 min prior to a ‘Weak’ tetanus prolonged the duration of the potentiation in the ‘Weak’ (S2) pathway. Without prior ‘Strong’ tetanisation, a ‘Weak’ tetanus results in decremental E-LTP.

Independent samples t tests showed that a pathway which received a ‘weak’ tetanus alone (i.e., the ‘weak’ alone experiments reported in Chapter 4) was not significantly different from the S2 pathway in these ‘Strong before Weak’ experiments at 10 min post-tetanus. Analysis of the ‘weakly’ tetanised pathways alone from each of these experiments showed significant differences at all later times (60, 180 and 360 min post-tetanus). Therefore, these results demonstrate that the persistence of LTP can be modulated by the prior stimulation of a convergent input to the same group of CA1 neurons. These results are summarised in Table 5.3. The next group of experiments addressed whether or not a similar effect could be seen when the order of ‘strong’ and ‘weak’ stimulation was reversed.

Figure 5.2 ‘Synaptic tagging’: Rescue of decremental LTP by prior ‘strong’ heterosynaptic stimulation.

- a. An individual experiment showing a representative example of the rescue of LTP resulting from ‘Weak’ tetanisation to S2, 50 min after a prior ‘Strong’ tetanus to S1.
- b. Grouped data for ‘Strong’ before ‘Weak’ experiments (n=6). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. Baseline (100 %) was taken as the mean of the values in consecutive 10min periods (i.e. 5 successive evoked responses), and 60min of pre-tetanus baseline response was recorded before three 100Hz tetanic trains (indicated by filled arrowhead) were delivered to S1. 30 min after the last of the three trains (i.e., 50 min after the first train) a single tetanus of 11 pulses at 100 Hz was delivered to S2 (indicated by empty arrowhead). Error bars indicate \pm s.e.m. Symbols: S1 strong tetanus pathway ●. S2 weak tetanus pathway ○.

a.



b.

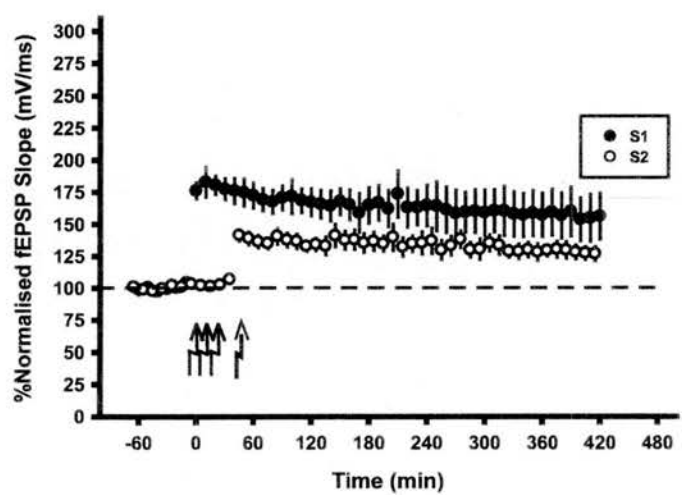


Table 5.3 Summary of statistical comparisons for ‘Weak’ alone vs. ‘Strong before Weak’ experiments.

Time (min)	‘Weak alone’ S1 pathway normalised EPSP slope	‘Strong before Weak’ S1 pathway normalised EPSP slope	t value	
-30	102.74 ± 1.00	100.54 ± 1.96	< 1	p > 0.30
10	131.61 ± 5.32	138.98 ± 4.99	< 1	p > 0.35
60	119.64 ± 4.82	136.61 ± 5.71	2.28	p < 0.05
180	108.59 ± 3.61	134.69 ± 6.41	3.78	p < 0.01
360	92.38 ± 3.86	127.25 ± 5.07	5.58	p < 0.005

Table 5.3: Independent sample t-test comparisons of S1 pathways, at a number of times before and after either a ‘Weak’ tetanus alone, or a ‘Weak’ tetanus after a ‘Strong’ tetanus. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

5.4.1 'Weak before Strong' experiments.

Similar tetanus protocols as used in the previous set of experiments were used in these experiments. The exception being that the 'weak' tetanus of 11 pulses was delivered 50 min before the repeated 'strong' high frequency tetanus.

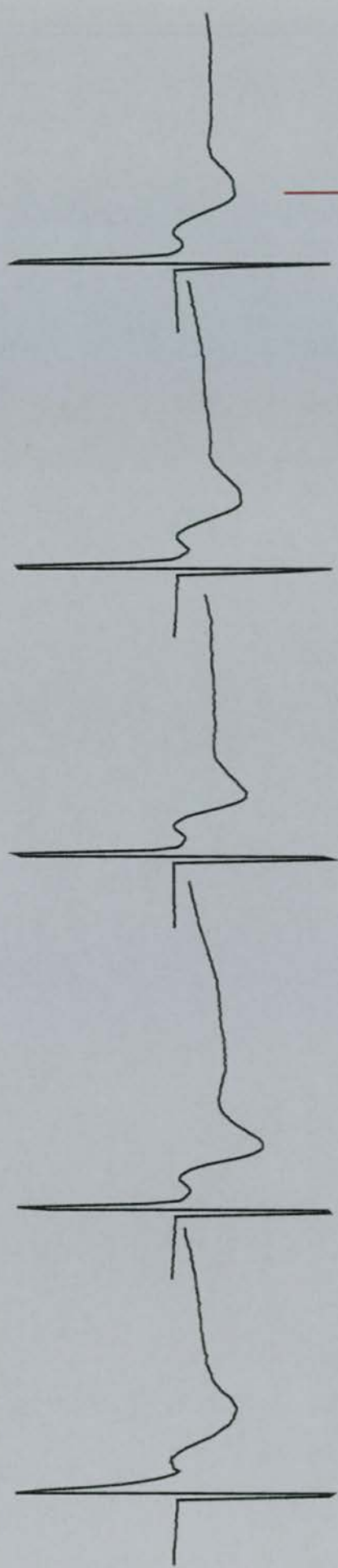
The averaged response over a range of stimulation intensities from 10 – 90 μ A increased from 0.53 to 3.17 mV/ms for S1, and from 0.12 to 3.17 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

Representative field EPSPs are shown from an individual 'weak before strong' experiment in Figure 5.4. There was a small amount of PTP in the S2 pathway (which was subsequently given 'strong' stimulation) after delivery of the 'weak' tetanus to the S1 pathway (fEPSP Slope = $107.25\% \pm 2.50$ s.e.m., $t = 2.90$, $p < 0.05$) compared to the baseline level. This largely reflects the fact that the pathways were unstable before the tetanus, with some drift in the response to test stimulation. The S1 pathway showed robust PTP after the 'weak' tetanus (fEPSP Slope = $174.61\% \pm 8.42$ s.e.m., $t = 8.86$, $p < 0.005$). Subsequent 'strong' tetanisation of S2 resulted in significant PTP in this pathway after the tetanus (fEPSP Slope = $191.16\% \pm 9.54$ s.e.m., $t = 9.55$, $p < 0.005$).

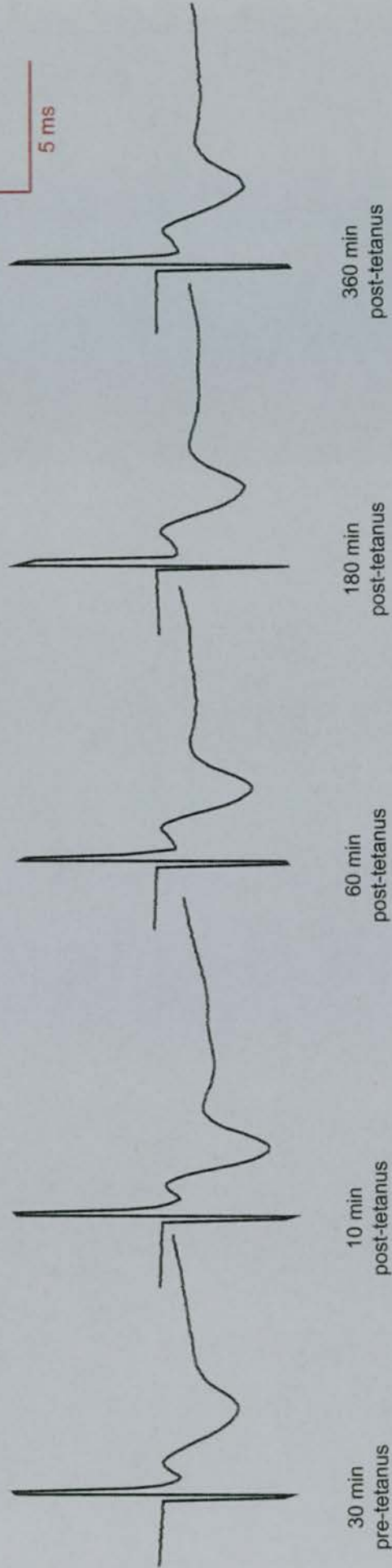
Figure 5.4 Representative fEPSPs waveforms from an individual ‘Weak before Strong’ experiment.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 5.5 a.

S1 - 'Weak' tetanus pathway



S2 - 'Strong' tetanus pathway



5.4.2 'Weak before strong' results in the transformation of E-LTP into L-LTP in a pathway given brief high frequency stimulation.

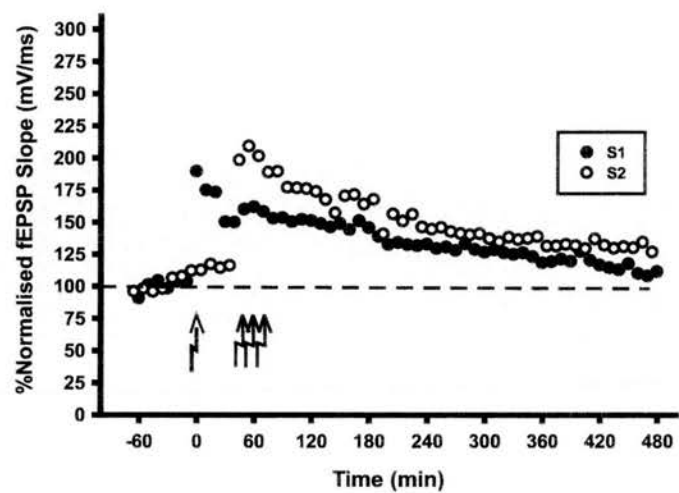
An individual example of an experiment where the S1 pathway was given 'weak' tetanic stimulation 50 min before S2 received 'strong' stimulation is shown in Figure 5.5 a. Stable baseline responses were recorded for 1 hr before S1 was tetanised ('weak' stimulation). This 'weak' tetanus induced LTP, with the level of potentiation being 174.60 % 10 min post-tetanus, and this remained elevated at 119.97 %, 420 min post-tetanus. The 'strong' tetanus resulted in an initial potentiation of 208.88 %, 10 min after this tetanus (i.e., 60 min after the 'weak' tetanus), and this remained at an elevated level for the entire post-tetanus period (136.57 % at 420 min).

Grouped data for 'Weak before Strong' experiments (n=6) are shown in Figure 5.5 b. In order to determine whether or not 'Strong' tetanisation had any effect on the duration of the potentiation induced by a prior 'Weak' tetanus, comparisons were made between these 'Weak before Strong' series of experiments, and experiments in which 'Weak' stimulation alone was delivered to just one pathway. A repeated measures ANOVA with time (10, 60, 180, 360 min) after the 'weak' tetanus as a within-subjects factor and experimental condition ('Weak' tetanus alone or 'Weak before Strong') as a between-subjects factor was conducted. There was a significant difference between the experiments [$F(1, 12) = 11.45$; $p < 0.01$] indicating that a 'Strong' tetanus delivered 50 min after a 'Weak' tetanus prolonged the duration of the potentiation in the 'Weak' (S2) pathway. Without the 'Strong' tetanisation, a 'Weak' tetanus results in a decremental form of LTP. Independent samples t tests showed that a pathway which received a 'weak' tetanus alone (i.e., the 'weak' alone experiments reported in Chapter 5) was significantly different from the S1 pathway from the 'Weak before Strong' experiments at all times post-tetanus. These results are summarised in Table 5.6.

Figure 5.5 ‘Synaptic tagging’: Rescue of decremental LTP by subsequent ‘strong’ heterosynaptic stimulation.

- a. An individual experiment showing a representative example of the rescue of LTP resulting from ‘Weak’ tetanisation to S1, 50 min before subsequent ‘Strong’ tetanus of S2.
- b. Grouped data for ‘Weak before Strong’ experiments (n=6). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. Baseline (100 %) was taken as the mean of the values in consecutive 10min periods (i.e. 5 successive evoked responses), and 60min of pre-tetanus baseline response was recorded before three 100Hz tetanic trains (indicated by filled arrowhead) were delivered to S1. 30 min after the last of the three trains (i.e., 50 min after the first train) a single tetanus of 11 pulses at 100 Hz was delivered to S2 (indicated by empty arrowhead). Error bars indicate \pm s.e.m. Symbols: S1 weak tetanus pathway ●. S2 strong tetanus pathway ○.

a.



b.

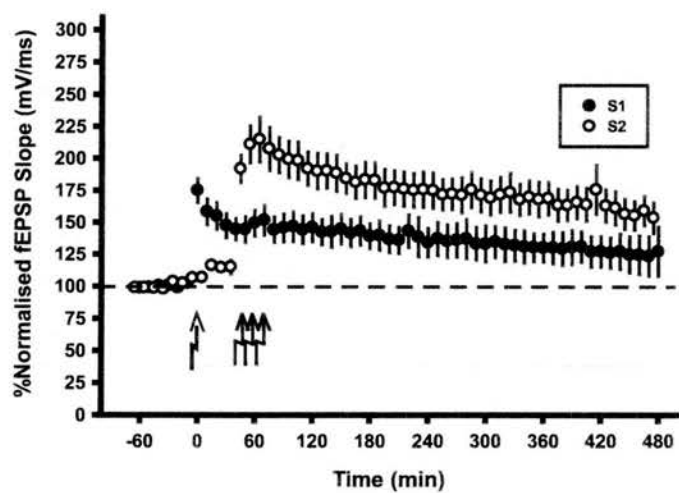


Table 5.6 Summary of statistical comparisons for ‘Weak’ alone vs. ‘Weak before Strong’ experiments.

Time (min)	‘Weak alone’ S1 pathway normalised EPSP slope	‘Weak before Strong’ S1 pathway normalised EPSP slope	t value	
-30	102.74 ± 1.00	100.49 ± 1.22	< 1	p > 0.1
10	131.61 ± 5.32	158.26 ± 10.03	2.52	p < 0.05
60	119.64 ± 4.82	149.31 ± 10.04	2.99	p < 0.05
180	108.59 ± 3.61	139.19 ± 9.78	3.27	p < 0.05
360	92.38 ± 3.86	130.15 ± 10.81	3.68	p < 0.05

Table 5.6: Independent sample t-test comparisons of S1 pathways, at a number of times before and after either a ‘Weak’ tetanus alone, or a ‘Weak’ tetanus before a ‘Strong’ tetanus.

5.4.3 Confounding factors in 'weak before strong' experiments.

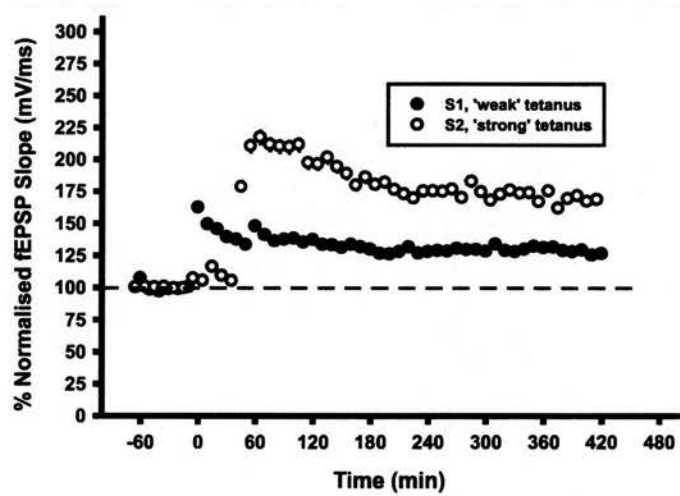
One possible confounding factor in the 'weak before strong' experiments is the apparent PTP in the S2 pathway following 'weak' stimulation of S1. If the pathways are not independent, one might expect to obtain heterosynaptic interactions between pathways, as was discussed in the introduction to this chapter. Unfortunately, it was not possible to test for independence of the pathways using a paired-pulse facilitation paradigm (as mentioned in Chapter 2 Materials & Methods). However, the effect was very small ($fEPSP = 107.25\% \pm 2.50$). It is likely that this was due to 'drift' in the response in this pathway prior to the 'strong' tetanus. A drift in the response of both pathways may explain why the initial level of potentiation obtained with the 'weak' tetanus (158.26 % at 10 min post-tetanus) in these 'weak before strong' experiments was greater than had been previously obtained with a 'weak' tetanus alone (131.61 % at 10 min post-tetanus), or the 'strong before weak' experiments (138.98 % at 10 min post-tetanus).

For statistical purposes, the results from six different experiments were pooled in order to allow comparisons to be made. Data was replotted from two experiments where little drift in the response was seen. These two experiments are shown in Figure 5.7, and the clearly demonstrate the 'rescue' of the potentiation in the 'weak' S1 pathway. The initial potentiation in the S1 pathway following a 'weak' tetanus was 149.20 % at 10 min, which again was greater than that found with a 'weak' tetanus alone, 131.61 % at 10 min post-tetanus. However, upon delivery of the 'strong' tetanus to S2 (at 50 min) it is clear that the response in S1 was influenced. Before the tetanus, the response in S1 was 133.27 % at 50 min post-tetanus, and this had increased to 147.72 % at 60 min post-tetanus (i.e., 10 min after the S2 'strong' tetanus). There was some PTP in the 'strong' pathway, ~ 108.52 %, after the 'weak' tetanus to S1, therefore some overlap between the pathways may have occurred. This may have occurred due to slight 'drift' in the response of the 'strong' tetanus pathway around the time of the 'weak' tetanus. However, without the ability to test for paired-pulse interactions between the two input pathways it is difficult to decide between these alternative interpretations.

Figure 5.7 Synaptic tagging with a ‘weak before strong’ experimental protocol.

- a. Data was plotted from two experiments where ‘weak’ stimulation was delivered to one pathway (S1), 50 min before ‘strong’ stimulation was delivered to an independent pathway (S2). These experiments showed no drift during the pre-tetanus baseline period, or following the ‘weak’ tetanus to S1, which may have been a confounding factor in the experiments shown in Figure 5.5.

a.



5.5.1 Protein synthesis and LTP.

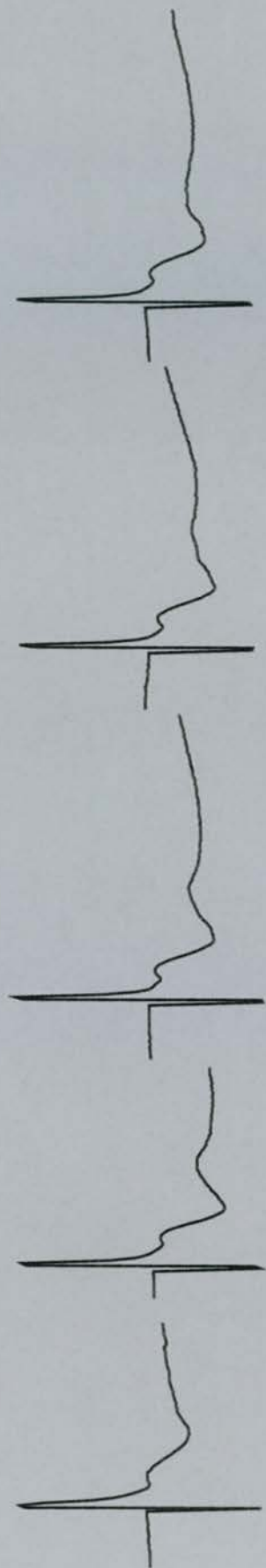
In the first series of experiments, anisomycin (25 and 50 μ M) was bath applied to hippocampal slices 30 min prior, and 60 min after high frequency stimulation.

For both experiments with 25 and 50 μ M anisomycin there was no significant difference between pathways in the response to stimulation across all intensities during the input/output measurement [$F < 1$]. Representative field EPSPs are shown from individual experiments with 25 μ M anisomycin in Figure 5.8., and with 50 μ M Anisomycin in Figure 5.9. There was no PTP in the control pathway after delivery of the first of the three tetani in the presence of 25 μ M anisomycin (fEPSP Slope = $107.15\% \pm 4.99$ s.e.m., $t = 1.43$, $p > 0.20$) compared to the baseline level. The tetanised pathway showed robust PTP (fEPSP Slope = $236.65\% \pm 17.10$ s.e.m., $t = 8.00$, $p < 0.005$). There was also no PTP in the control pathway after delivery of the first of the three tetani (fEPSP Slope = $103.39\% \pm 8.77$ s.e.m., $t = 0.39$, $p > 0.71$) in the presence of 50 μ M anisomycin, compared to the baseline level. The tetanised pathway again showed robust PTP (fEPSP Slope = $232.66\% \pm 31.51$ s.e.m., $t = 4.21$, $p < 0.01$).

Figure 5.8 Representative fEPSPs waveforms from an individual experiment with 25 μ M anisomycin.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 5.10 a.

S1 - Tetanised pathway



S2 - Non-tetanised pathway

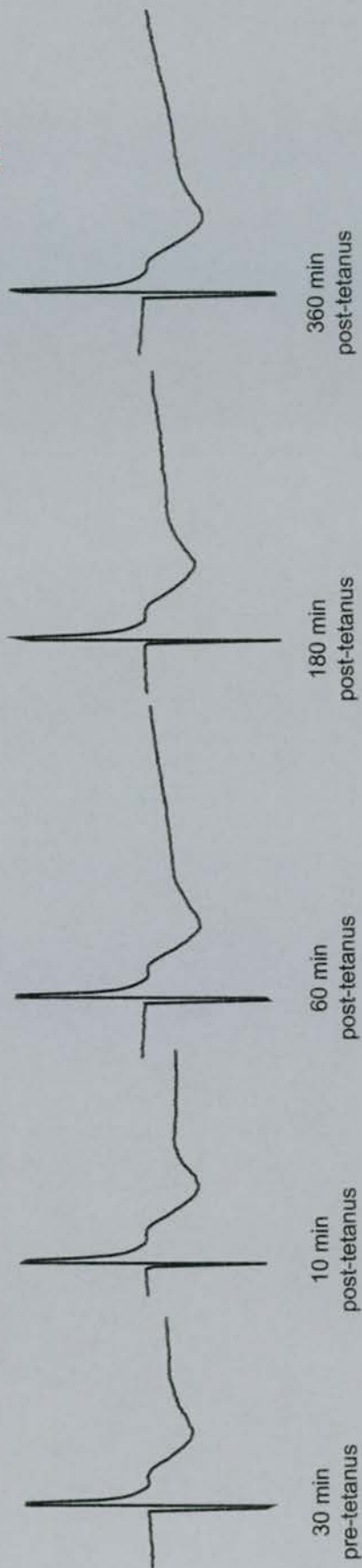
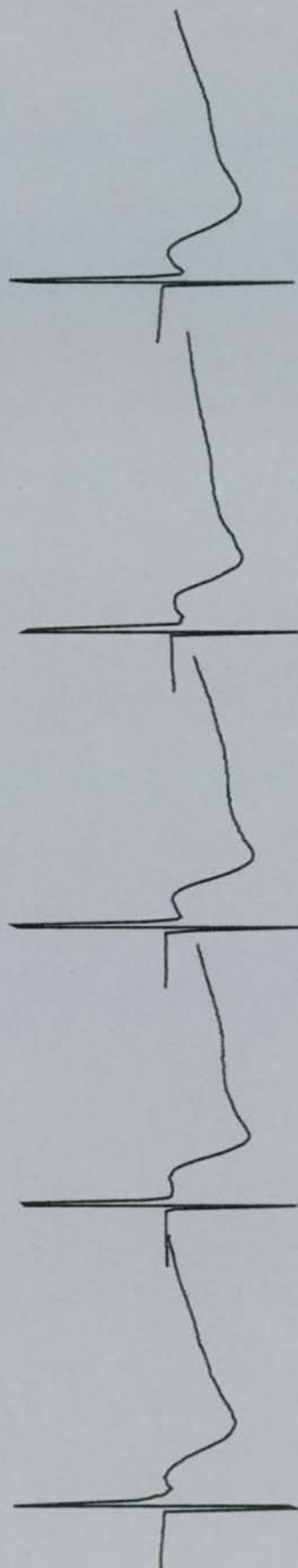


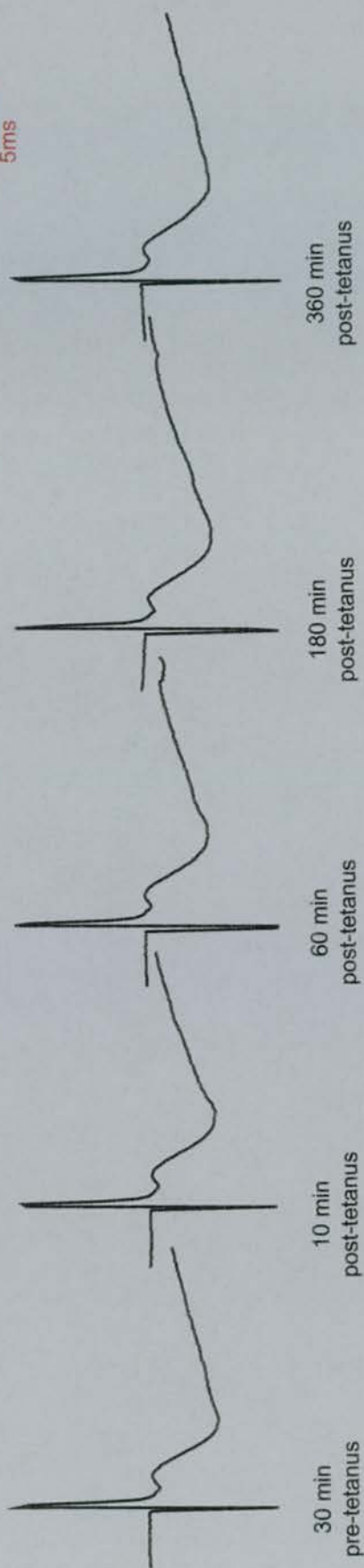
Figure 5.9 Representative fEPSPs waveforms from an individual experiment with 50 μ M anisomycin.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 5.11 a.

S1 - Tetanised pathway



S2 - Non-tetanised pathway



5.5.2 L-LTP is not affected by anisomycin.

Individual examples of experiments with 25 μ M anisomycin are shown in Figure 5.10 a and for 50 μ M anisomycin in Figure 5.11 a. In both experiments the drug was applied to the slice 30 min before the tetanus, and washed of 60 min after the first of the three trains (indicated on each graph). For both experiments with 25 μ M and 50 μ M anisomycin, robust LTP was induced that was non-decremental over the entire post-tetanus period. The control pathways (S2) from both groups of experiments showed no change during the experiment.

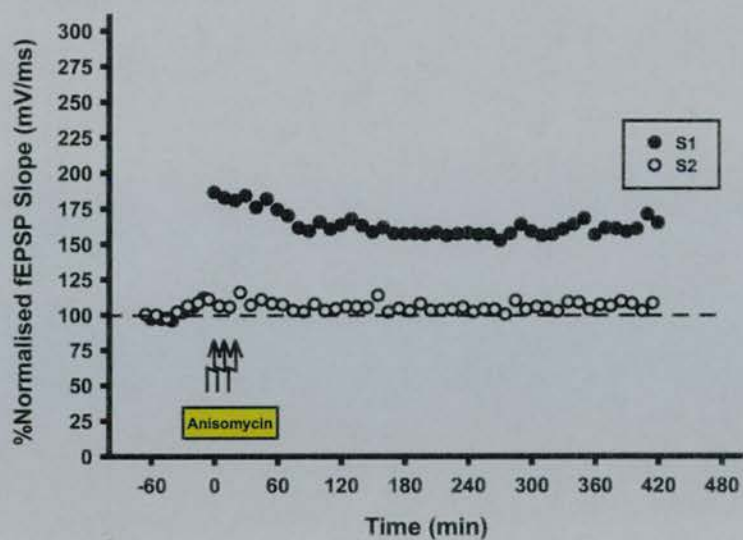
The results of experiments with 25 μ M anisomycin ($n=7$) are shown in Figure 5.10 b and with 50 μ M anisomycin ($n=6$) in Figure 5.11 b. Separate repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors were conducted for both groups of experiments. With either 25 or 50 μ M anisomycin in the bath, 'strong' stimulation induced robust L-LTP. Statistical analysis showed that this was the case with 25 μ M anisomycin [$F(1, 6) = 25.6$; $p < 0.01$], and 50 μ M anisomycin [$F(1, 5) = 23.6$; $p < 0.01$]. In both groups of experiments, significant interactions were found between pathway and time [$F(3, 18) = 9.40$; $p < 0.01$] for 25 μ M, [$F(3, 15) = 16.41$; $p < 0.005$] for 50 μ M. This is explained by a small decline in response in the tetanised pathway (S1) in both the 25 μ M experiments [$F(3, 18) = 6.91$; $p < 0.01$], and the 50 μ M experiments [$F(3, 15) = 13.39$; $p < 0.005$]. There was no significant change in the control pathway (S2) for either the 25 μ M group [$F(3, 18) = 1.20$; $p > 0.05$], or the 50 μ M group [$F(3, 15) = 0.15$; $p > 0.05$]. Statistical comparisons (paired samples t tests) are shown in Tables 5.13 a + b.

Comparing these experiments with anisomycin (see Figure 5.12) clearly indicates that L-LTP was unaffected by the application of anisomycin at either concentration to hippocampal slices. For all groups, significant potentiation was maintained for up to 6 hr post-tetanus, and the magnitude of potentiation did not differ between groups.

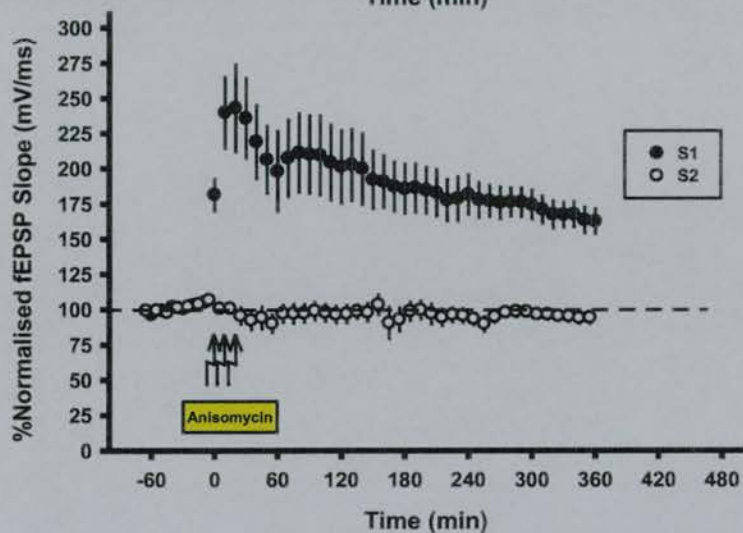
Figure 5.10 L-LTP is unaffected by 25 μ M anisomycin.

- a. An individual experiment showing a representative example of LTP in the presence of 25 μ M anisomycin. The drug was applied 30 min before three high frequency tetanic trains (indicated by arrows) to S1 pathway, and remained until 60 min after the first of the three trains. The yellow bar shows the application of the drug.
- b. Grouped data for experiments ($n=7$) in which L-LTP was induced in the presence of the translational inhibitor anisomycin (25 μ M). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus level is shown. Basal responses were recorded for 60 min before three 100Hz tetanic trains (indicated by arrows) were delivered to S1 at 10 min intervals. Error bars indicate \pm s.e.m. Symbols: S1 tetanised pathway \bullet . S2 non-tetanised pathway \circ .
- c. Statistical comparisons were made between the tetanised and the non-tetanised control pathway both before, and after the tetanus in the presence of 25 μ M anisomycin. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at each time point. * indicates a statistically significant difference with $p < 0.05$. (Paired students t-test, Bonferroni correction for multiple comparisons).

a.



b.



c.

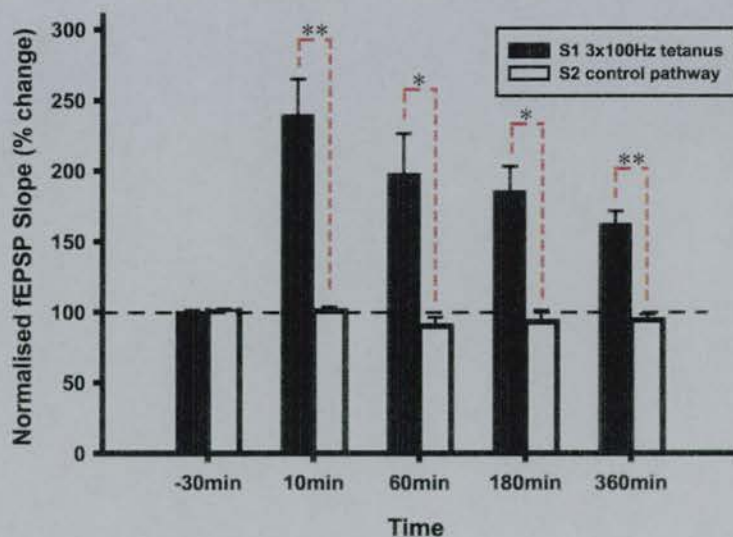


Figure 5.11 L-LTP is unaffected by 50 μ M anisomycin.

- a. An individual experiment showing a representative example of LTP in the presence of 50 μ M anisomycin. The drug was applied 30 min before three high frequency tetanic trains (indicated by arrows) to S1 pathway, and remained until 60 min after the first of the three trains. The yellow bar shows the application of the drug.
- b. Grouped data for experiments (n=6) in which L-LTP was induced in the presence of the translational inhibitor anisomycin (50 μ M). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus level is shown. Basal responses were recorded for 60 min before three 100Hz tetanic trains (indicated by arrows) were delivered to S1 at 10 min intervals. Error bars indicate \pm s.e.m. Symbols: S1 tetanised pathway ●. S2 non-tetanised pathway ○.
- c. Statistical comparisons were made between the tetanised and the non-tetanised control pathway both before, and after the tetanus in the presence of 50 μ M anisomycin. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at each time point. * indicates a statistically significant difference with $p < 0.05$. (Paired students t-test, Bonferroni correction for multiple comparisons).

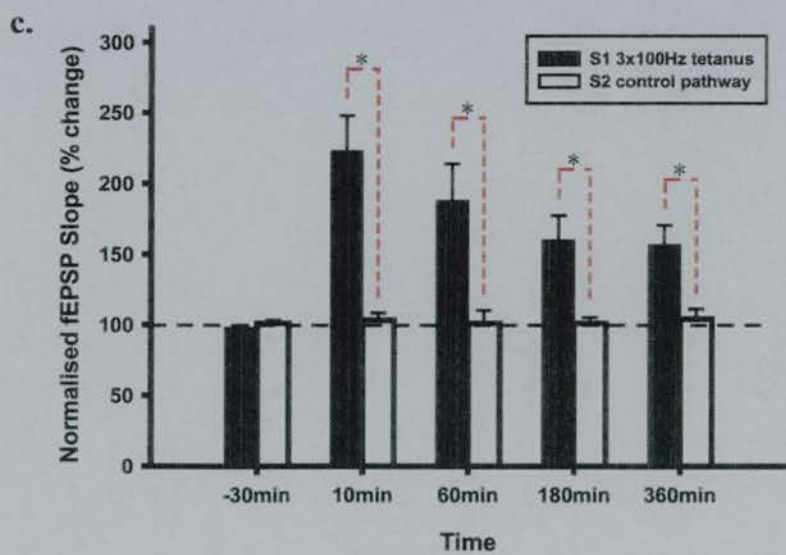
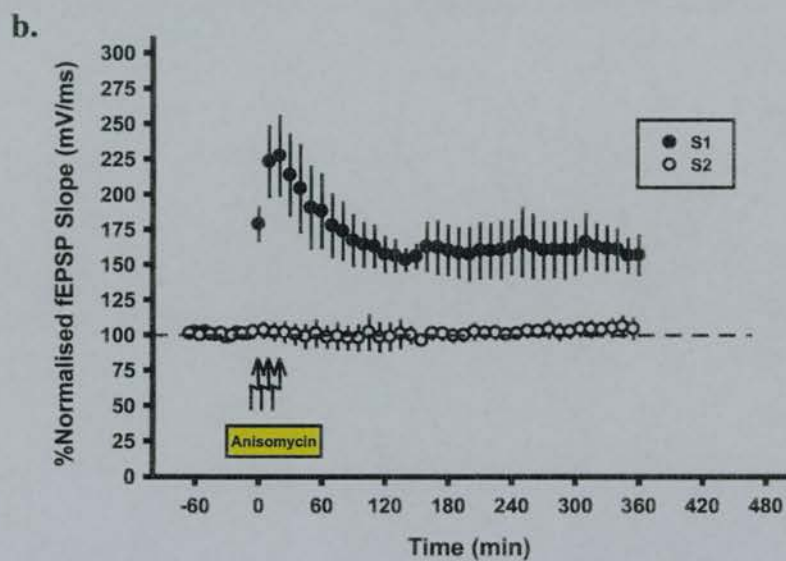
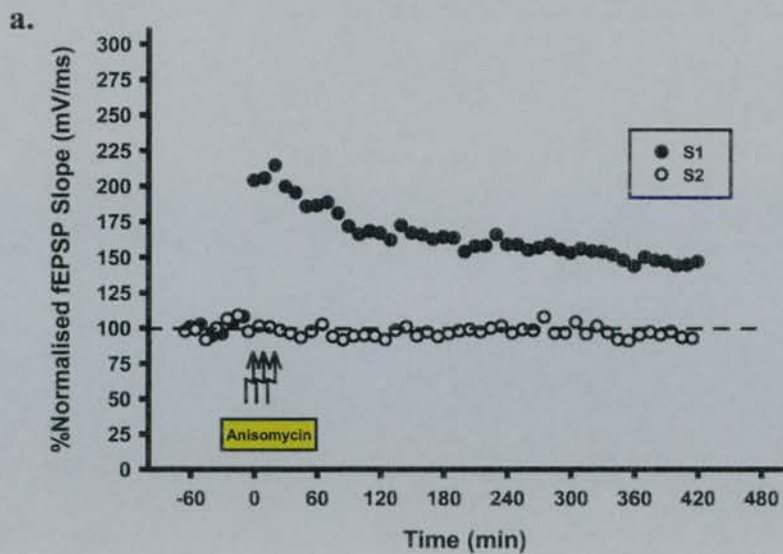


Figure 5.12 L-LTP in CA1 is unaffected by the application of anisomycin.

- a. Comparing the control experiments where no drug was applied to the slices, to experiments where 25 μ M anisomycin was bath applied shows that L-LTP was induced in both groups.
- b. Comparing the control experiments where no drug was applied to the slices, to experiments where 50 μ M anisomycin was bath applied shows that L-LTP was induced in both groups. These control experiments ($n = 12$) were presented in Chapter 3, however from this group of twelve, four experiments were interleaved with the anisomycin experiments, two for each concentration of the drug. These four 'control' experiments conducted during the anisomycin experiments were no different from the overall data set in the series.

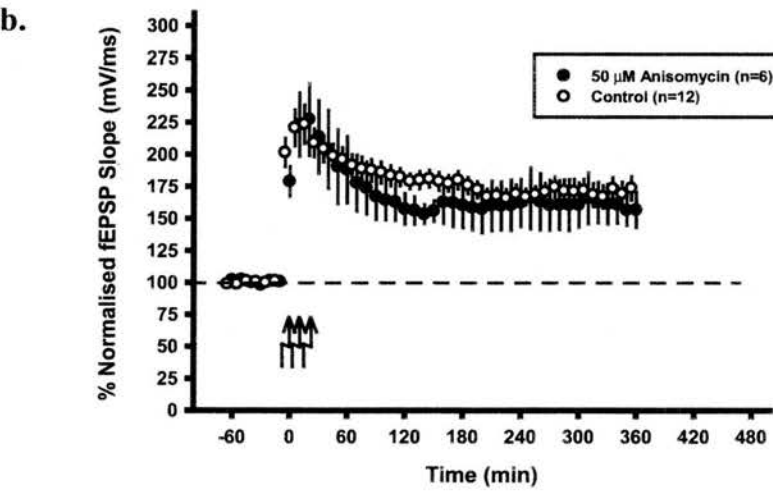
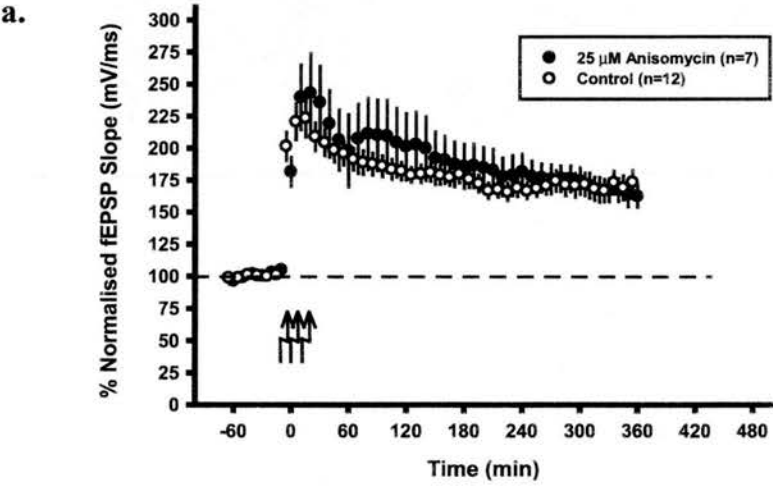


Table 5.13 Summary of statistical comparisons for 25 μ M and 50 μ M anisomycin experiments.a. 25 μ M anisomycin.

Time (min)	S1 pathway normalised EPSP slope	S2 pathway normalised EPSP slope	t value	
-30	100.21 \pm 1.21	101.07 \pm 1.13	0.54	p >0.05
10	239.72 \pm 25.18	100.60 \pm 3.05	5.41	p <0.01
60	197.92 \pm 28.38	92.83 \pm 4.73	3.84	p <0.05
180	185.63 \pm 17.39	99.99 \pm 4.15	4.78	p <0.05
360	162.50 \pm 8.81	94.06 \pm 4.22	6.56	p <0.01

b. 50 μ M anisomycin.

Time (min)	S1 pathway normalised EPSP slope	S2 pathway normalised EPSP slope	t value	
-30	98.00 \pm 1.47	100.98 \pm 2.30	0.40	p >0.05
10	223.09 \pm 24.79	103.22 \pm 5.19	5.34	p <0.05
60	187.92 \pm 26.01	99.36 \pm 8.10	4.31	p <0.05
180	160.07 \pm 17.29	100.93 \pm 4.21	4.00	p <0.05
360	156.55 \pm 13.84	100.90 \pm 4.28	4.99	p <0.05

Table 5.13: Paired samples t-test comparisons of S1 and S2 pathways, at a number of times before and after repeated high frequency stimulation in the presence of either 25 or 50 μ M anisomycin. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

5.6.1 Does emetine block L-LTP?

As anisomycin was found to have no inhibitory effect on L-LTP, the protein synthesis inhibitor emetine was tested.

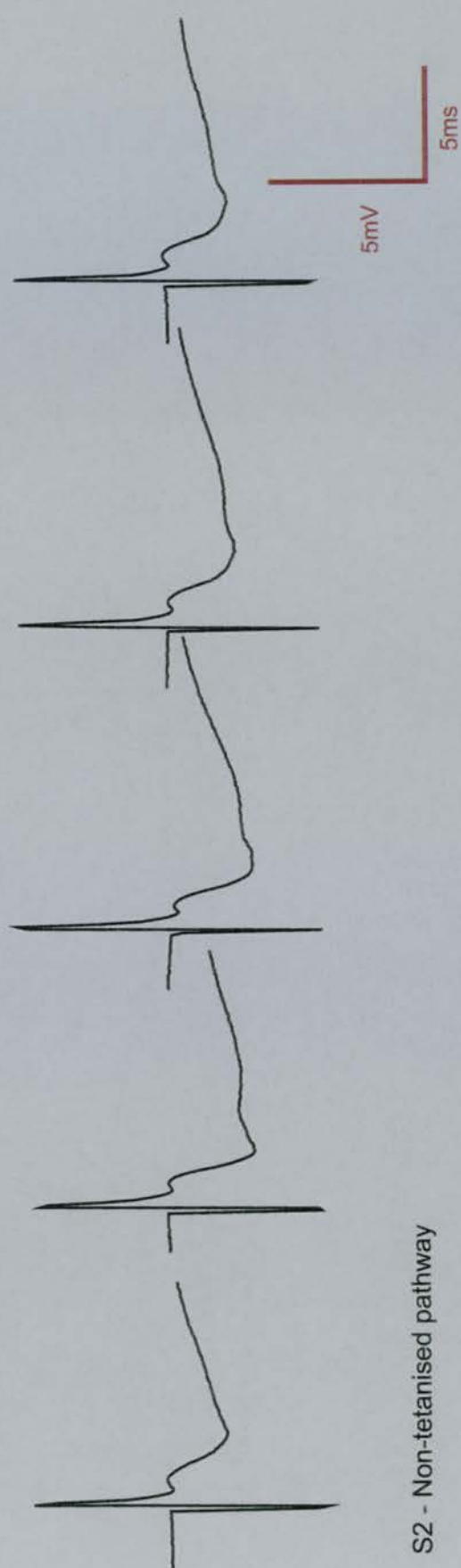
The averaged response over a range of stimulation intensities from 10 – 90 μ A increased from 0.60 – 3.10 mV/ms for S1, and from 0.18 – 3.23 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

Representative field EPSPs are shown from an individual experiment in Figure 5.14. There was significant PTP in the control pathway after delivery of the first of the three tetani (fEPSP Slope = $134.74 \% \pm 3.63$ s.e.m., $t = 9.57$, $p < 0.005$) compared to the baseline level. The tetanised pathway showed robust PTP (fEPSP Slope = $207.80 \% \pm 6.40$ s.e.m., $t = 16.85$, $p < 0.005$). The reason for this change in the baseline response in the control pathway is due to the fact that application of emetine caused some non-specific effects on the baseline response evoked from both pathways. This was addressed by a series of control experiments that are reported in a later section.

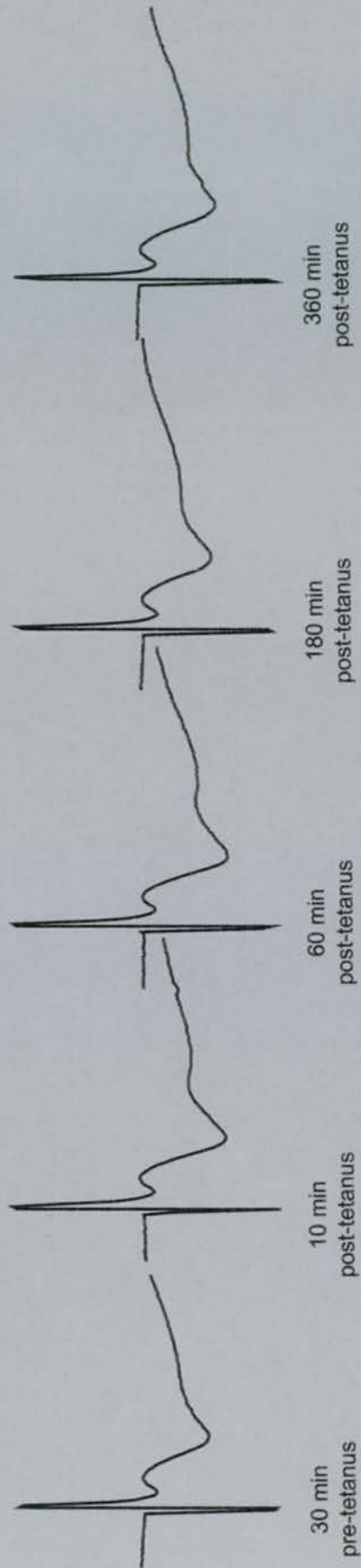
Figure 5.14 Representative fEPSPs waveforms from an individual experiment with 100 μ M emetine.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 5.15 a.

S1 - Tetanised pathway



S2 - Non-tetanised pathway



5.6.2 The effect of emetine on L-LTP.

An individual example of one of the experiments, which form part of the grouped data presented later, is shown in Figure 5.15 a. The tetanus was delivered to the S1 pathway, 10min post-tetanus the response was 178.54 % of the pre-tetanus baseline level. The level of potentiation was 121.87 % at 360min post-tetanus. The control pathway (S2) showed a small change from 125.30 % at 10 min, to 97.94 % at 60 min post-tetanus.

The results of experiments with 100 μ M emetine (n=8) are shown in Figure 5.15 b. L-LTP was not completely blocked in the presence of 100 μ M emetine, even though the drug did seem to have some effect on the magnitude of potentiation. The tetanised pathway (S1) was still elevated at 6 hr post-tetanus, with no change in the S2 control pathway. Interleaved control experiments conducted during these experiments with emetine showed that L-LTP was greater in magnitude when the drug was not present. The interleaved controls (n = 2) are shown in Figure 5. 15 c. The LTP recorded in these interleaved controls was not different from that reported with identical experiments shown in Chapter 3 enabling later comparisons to be made between these ‘control’ experiments and the emetine treated experiments.

However, focusing on just the experiments with emetine, a repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors was conducted. There was a significant main difference between the pathways [$F(1, 7) = 289.04$; $p < 0.005$], indicating that the tetanus produced sustained L-LTP even after application of emetine to the slices. A significant pathway x time interaction was found [$F(4, 28) = 44.57$; $p < 0.005$]. Subsequent analysis revealed a main effect of time in both the tetanised pathway (S1) [$F(4, 28) = 76.35$; $p < 0.005$], and the control pathway (S2) across these intervals [$F(4, 28) = 29.00$; $p < 0.001$], reflecting the fact that the basal response changed as a result of application, and subsequent washing out, of the drug. Statistical comparisons (Paired samples t tests with Bonferroni correction for multiple comparisons), between the tetanised and the control pathways at various time points during the experiment showed significant differences at all times, these results are presented in Table 5.16.

Figure 5.15 L-LTP is not completely blocked by 100 μ M emetine.

- a. An individual experiment showing a representative example of LTP in the presence of 100 μ M emetine. The drug was applied 30 min before three high frequency tetanic trains (indicated by arrows) to S1 pathway, and remained until 60 min after the first of the three trains. The yellow bar shows the application of the drug.
- b. Grouped data for experiments ($n=8$) in which L-LTP was induced in the presence of the translational inhibitor emetine (100 μ M). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus level is shown. Basal responses were recorded for 60 min before three 100Hz tetanic trains (indicated by arrows) were delivered to S1 at 10 min intervals. Error bars indicate \pm s.e.m. Symbols: S1 tetanised pathway \bullet . S2 non-tetanised pathway \circ .
- c. Grouped data for experiments ($n=2$) in which L-LTP was induced by three high frequency trains. These experiments were interleaved with the experiments with emetine show in Figure 5.16 b. Symbols: S1 pathway \bullet ; S2 pathway \circ .

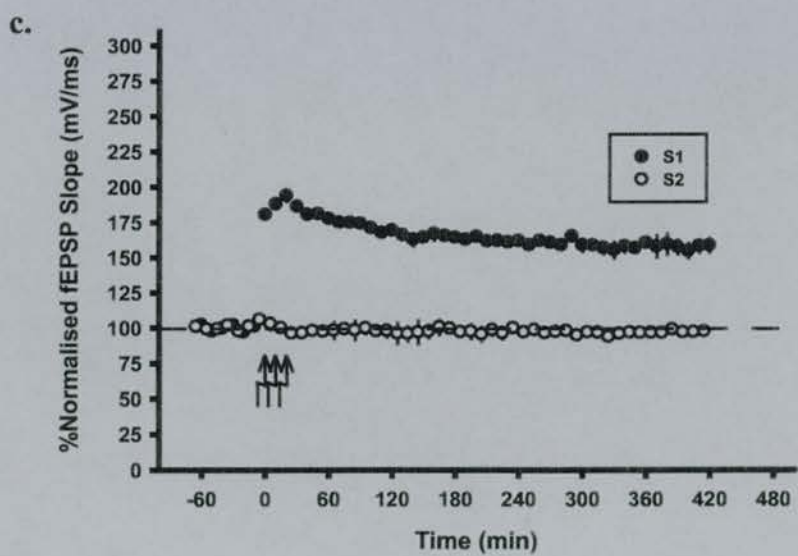
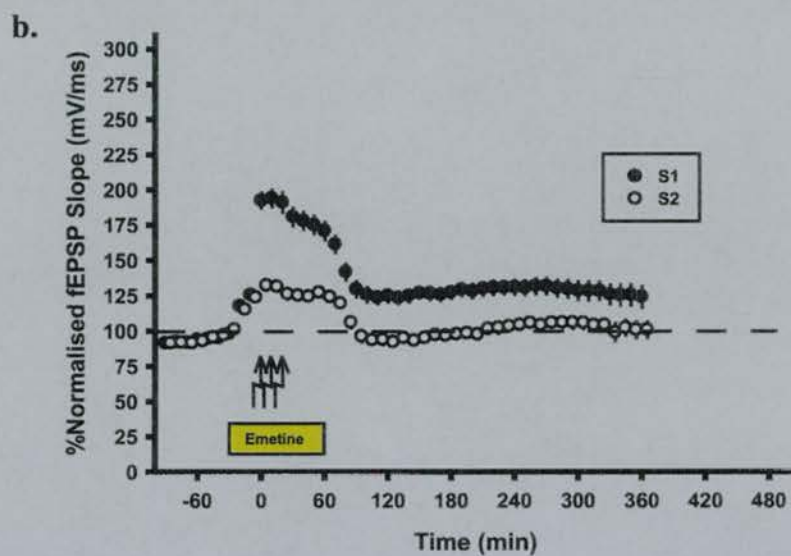
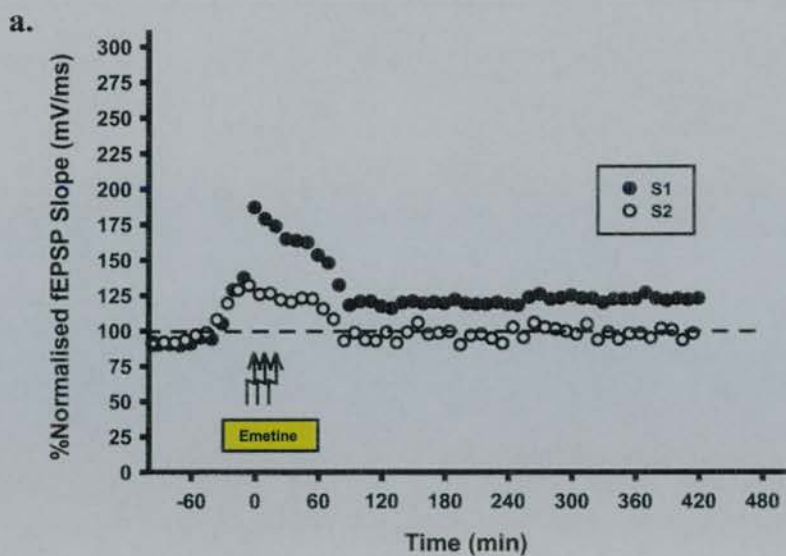


Table 5.16 Summary of statistical comparisons for 100 μ M emetine experiments.

Time (min)	S1 pathway normalised EPSP slope	S2 pathway normalised EPSP slope	t value	
-30	98.00 \pm 1.70	101.02 \pm 1.44	< 1	p > 0.05
10	194.02 \pm 5.96	131.42 \pm 3.52	15.9	p < 0.005
60	170.81 \pm 6.40	124.07 \pm 2.20	9.2	p < 0.005
180	127.18 \pm 4.45	97.60 \pm 3.35	8.7	p < 0.005
360	123.93 \pm 7.50	100.72 \pm 5.74	5.4	p < 0.005

Table 5.16: Paired samples t-test comparisons of S1 and S2 pathways, at a number of times before and after repeated high frequency stimulation in the presence of either 100 μ M emetine. For all analyses, the p values were adjusted according to Bonferroni's t correction for multiple comparisons. Significant differences were observed at all times post-tetanus.

5.6.3. Control experiments with application of emetine during test stimulation alone.

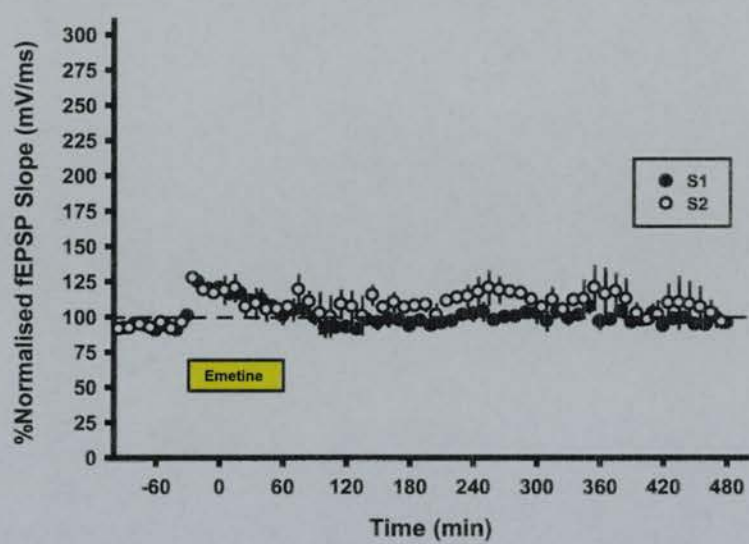
It is possible that emetine had an effect on a number of parameters such as membrane potential, input resistance, action potential amplitude or duration, IPSP amplitude, the threshold for evoking an action potential, or the Ca^{2+} dependent potassium afterhyperpolarization, which may account for the change in baseline test responses upon application of the drug to the slice. It is difficult to distinguish with field recordings if this was the case. However with intracellular recordings Stanton and Sarvey, (1984) did not find any such effects of emetine and no effects were observed in the field recordings.

As can be seen from Figure 5.15 b., application of this drug to hippocampal slices resulted in an increase in the basal response of $\sim 25\%$, compared to the normalised level before application of the drug. As this was a consistent finding with this drug, control experiments ($n=2$) were performed in which the drug was washed on for the same time period as before (i.e., 30 min before the tetanus, until 60 min after the last tetanus), without the delivery of any high frequency stimulation to either pathway, only test pulses were delivered. The results of these experiments are shown in Figure 5.17. A similar 'wash-on/wash-off' effect was found in these experiments, indicating that this drug appeared to produce effects on the evoked response that reversed upon washout of the drug. The response from both the S1 and S2 pathways were found to increase by $\sim 20\%$ when at the time the drug reaches the slice (-30 min). The mechanism by which this occurs is not clear.

Figure 5.17 Emetine shows some acute effects on baseline test responses upon application of the drug to hippocampal slices.

Data from control experiments (n=2) where emetine (100 μ M) was applied during basal test stimulation alone, neither pathway was tetanised in these experiments. The timing and duration of drug application is identical to that used in previous experiments with high frequency stimulation. The yellow bar represents the application of the drug.

a.



5.6.4 L-LTP with 100 μ M emetine is less robust than that induced with high frequency tetani alone.

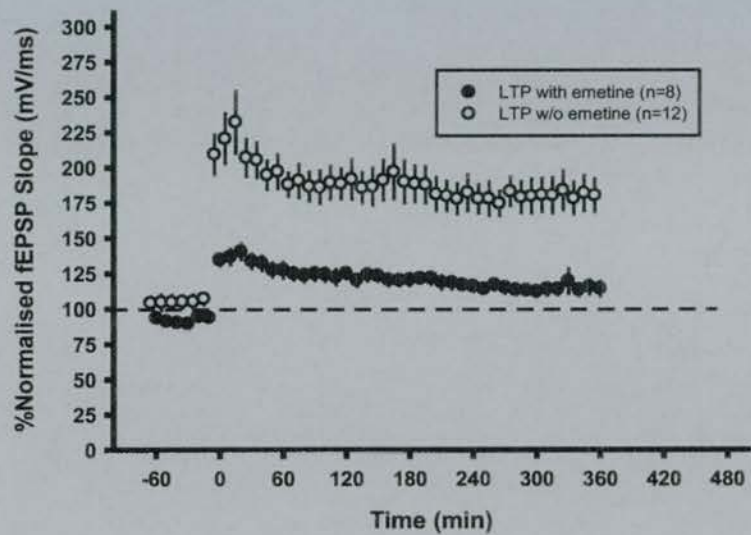
Rather than normalising the data to the pre-tetanus 60 min baseline response, the pathways (S1 and S2) were normalised to each other for the entire duration of the experiment, thus subtracting out the ‘wash on/off’ effect of drug application. This is presented in Figure 5.18 a. In addition to normalising the emetine data in this manner, the control experiments without any drug (that were presented in Chapter 3.) were also replotted using the same normalising function to allow comparison. The presence of emetine in the bath clearly affects the LTP when plotted this way.

Comparing this re-normalised data for experiments with and without emetine, LTP was significantly less in the presence of emetine. The results are presented in Figure 5.18 b. A repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors and drug treatment (high frequency tetanus in the presence, or absence, of emetine) as a between-subjects factor was conducted. There was a significant difference between experiments [$F(1, 18) = 19.11$; $p < 0.005$] which indicates that significantly less potentiation was induced in the presence of emetine. A significant effect of time [$F(3, 54) = 9.25$; $p < 0.005$] shows that both groups differed across all times post-tetanus. Further analysis with independent samples t tests showed that slices treated with emetine were significantly different from the controls at all times, as shown in Figure 5.18 b and summarised in Table 5.19.

Figure 5.18 In the presence of emetine, L-LTP is significantly smaller in magnitude at 6 hr post-tetanus.

- a. The data was re-plotted by normalising the S1 tetanus path to the S2 tetanus path (rather than to the pre-tetanus baseline as before) for both the drug treated group and the 'control' experiments where high frequency stimulation was delivered without emetine during the tetanus. Symbols: ● = re-normalised tetanus path with emetine (n=8). ○ = re-normalised tetanus path without emetine (n=12).
- b. Statistical comparisons were made between the tetanised pathways only, from experiments with and without the drug. *** indicates a statistically significant difference with $P < 0.005$, ** with $P < 0.01$ (Independent samples t-test, p values were adjusted according to Bonferroni correction for multiple comparisons). Error bars indicate \pm s.e.m. Symbols: Filled bars = high frequency stimulation without drug. Empty bars = high frequency stimulation with 100 μ M emetine.

a.



b.

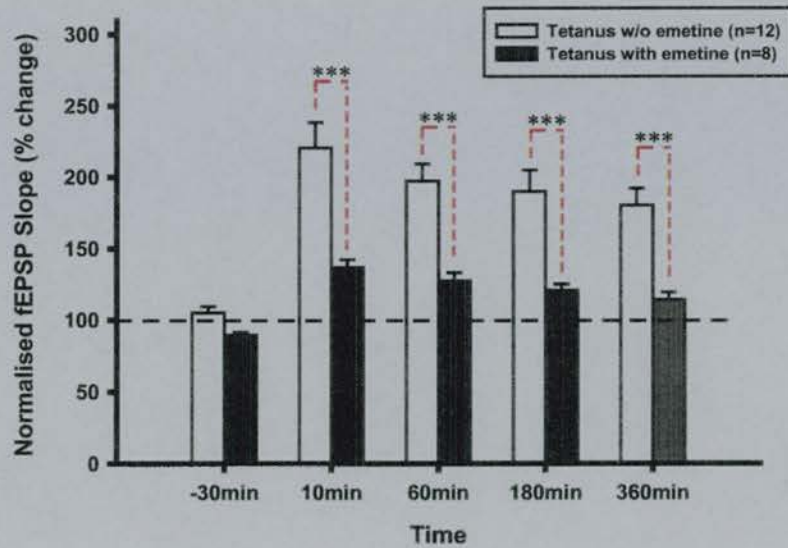


Table 5.19 Summary of statistical comparisons for ‘control’ vs. 100 μ M emetine experiments.

Time (min)	Emetine (100 μM) S1 re-normalised EPSP slope	Control (no drug) S1 re-normalised EPSP slope	t value	
10	136.80 \pm 5.24	220.52 \pm 14.06	3.74	p < 0.01
60	127.46 \pm 5.81	197.18 \pm 12.09	4.45	p < 0.005
180	120.66 \pm 3.94	189.86 \pm 15.01	3.68	p < 0.01
360	114.20 \pm 4.55	180.28 \pm 11.70	4.43	p < 0.005

Table 5.19: Independent samples t-test comparisons of S1 pathways, at a number of times after repeated high frequency stimulation in the presence of either aCSF or 100 μ M emetine. For all analyses, the p values were adjusted according to Bonferroni’s correction for multiple comparisons.

5.6.5 Emetine inhibits translation in hippocampal slices.

A number of experiments were undertaken to measure the incorporation of [35 S]-methionine in hippocampal slices as an assay of newly translated protein, and to determine whether this was inhibited by application of emetine (100 μ M). In order to address this a number of experiments were conducted and these are schematically illustrated in Figure 5.20.

In the first experiment, slice pairs were preincubated in the recording chamber for 2.5 hr as in the physiology experiments. Following this, one slice of each pair was removed from the chamber and transferred to an incubator where [35 S]-methionine was added to the aCSF. Emetine (100 μ M) was applied to the other slice in the recording chamber and this slice was incubated for 2 hr in the drug. The drug was then washed out for 30 min and then this slice was removed and [35 S]-methionine was added as before. Figure 5.21 A, shows the results from one 'control' slice (lane 1) and one emetine treated slice (lane 2). Regardless of whether the slice was treated with emetine for 2hr or not, clear bands representing newly translated proteins were labelled in both cases. In both cases proteins ranging from 200 kD to 20 kD are clearly visible.

In a second experiment, slices were preincubated for 2.5 hr in the recording chamber, and following this emetine (100 μ M) was added. The slices were incubated for a further 2 hr in emetine before one slice was removed and [35 S]-methionine was added as before. The other slice remained in the chamber and was perfused with emetine for a total of 8 hr before this slice was removed and [35 S]-methionine was added as before. In the first slice (2 hr emetine) of this pair, shown in Figure 5.21 B, newly translated proteins were labelled by [35 S]-methionine (lane 1). However, the slice incubated in emetine for 8 hr showed no bands of newly translated proteins (lane 2). Before removing both slices from the chamber field EPSPs were evoked from each slice (data not shown), indicating that both slices were viable at this time, most importantly the 8 hr treated slice. In addition to the slices protein translation in oocytes was assessed as a positive control for the labelling procedure and also to be confident that the drug was blocking translation. Figure 5. 21 B, shows that newly translated proteins ranging from 200 kD to 20 kD were labelled without emetine (lane 3), but there was some reduction, when emetine was applied for 3 hr (lane 4). The reduction in labelling proteins in the oocyte after a 3 hr incubation was not as complete as that seen in the slice treated for 8 hr.

Figure 5.20 Schematic representation of the experimental protocol in which protein translation in hippocampal slices was measured by the incorporation of [^{35}S]-methionine.

- a. In this experiment, one slice was incubated in aCSF for 2.5 hr (represented by #1 in the picture) before being removed from the chamber and placed in an incubator for 1 hr with [^{35}S -methionine] added to the solution. Immediately after removing the first slice, emetine (100 μM) was bath applied (represented by #3 coloured red in the picture) to the other slice and this slice was perfused with the drug for 2 hr before the drug was washed off for 30 min (represented by #2 in the picture). This slice was then removed and placed in an incubator for 1 hr with [^{35}S]-methionine added to the solution.
- b. In this experiment, both slices were incubated in aCSF for 2.5 hr (represented by #1 in the picture) before addition of emetine (100 μM) to the bath (represented by #3 coloured red in the picture). The drug was perfused for 2 hr before one slice was removed and placed in an incubator for 1 hr with [^{35}S]-methionine added to the solution as before. The other slice was left to perfuse in the recording chamber with emetine for a total of 8 hr (represented by #3 coloured red in the picture) before this slice was removed and placed in an incubator for 1 hr with [^{35}S]-methionine added to the solution.

a.



b.

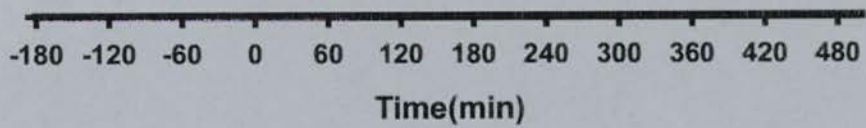
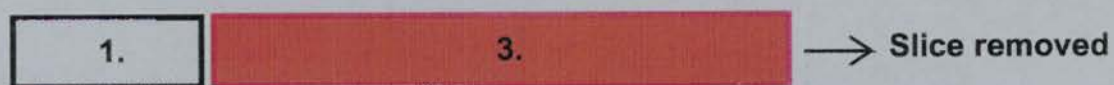


Figure 5.21 The incorporation of of [³⁵S]-methionine into hippocampal slices is blocked by prolonged incubation with emetine (100 μ M)

- A. SDS-PAGE denaturation and separation of brain proteins was conducted on all samples. Newly synthesised proteins were visualised by the incorporation of radiolabelled [³⁵S]-methionine. In the control slice left untreated, proteins ranging from ~ 200 kD to 20 kD are clearly visible (lane 1). Application of emetine (100 μ M) for 2 hr had no effect on the synthesis of new proteins; the slice treated in this way also showed incorporation of [³⁵S]-methionine (lane 2).
- B. As before, in a slice treated with emetine (100 μ M) for 2 hr, proteins ranging from ~ 200 kD to 20 kD are visible (lane 1). However, when emetine was applied for 8 hr, the synthesis of new proteins is blocked (lane 2). As a positive control for the labelling procedure, and the effectiveness of the emetine to inhibit translation, the exact same procedure was conducted using oocytes. In the control untreated oocytes, proteins ranging from ~ 200 kD to 20 kD are clearly visible (lane 3). This was reduced, although not completely abolished when emetine was applied for 3 hr (lane 4).

Figure 5.22 [³⁵S]-Methionine cell labelling



5.7 Discussion.

The results of the present experiments replicated one of the key findings of the original synaptic tag experiments of Frey and Morris, (1997, 1998a). They demonstrate that the persistence of LTP can be heterosynaptically modulated by stimuli occurring either before or after, the induction of LTP at a subset of the same group of CA1 neurons. This supports the idea that rather than the persistence of LTP being determined at the time of induction, the variable persistence of LTP can be influenced by events prior and subsequent to the induction of LTP at a subset of CA1 neurons.

5.7.1 *The heterosynaptic rescue of E-LTP.*

In chapter 4, experiments with a 21 pulse tetanus induced an LTP that was non-decremental for up to 6 hr post-tetanus, the initial level of potentiation in these experiments was 141.33 % at 10 min, and stabilised at 128.89 % at 6 hr post-tetanus. As was discussed in Chapter 4, it would be desirable to dissociate between the persistence of LTP being due to either some threshold of short-term potentiation having to be crossed to observe persistent LTP or, the initial magnitude of potentiation being independent of the temporal persistence of LTP. The results from all six ‘weak before strong’ experiments, may not allow a clear distinction to be made between these alternatives. However, as the data from two slices show, with more stable baseline responses L-LTP may be heterosynaptically ‘rescued’ by ‘strong’ high frequency stimulation. PTP was not observed in the ‘strong before weak’ series of experiments which lead to the same conclusion as the ‘weak before strong’ series, namely that a ‘strong’ tetanus can facilitate the persistence of LTP on an independent input pathway in the CA1.

5.7.2. *Why do anisomycin and emetine fail to block L-LTP?*

Results were presented from experiments in which the protein synthesis inhibitor anisomycin was applied to hippocampal slices both during a time window, and at a concentration, which has been previously shown to block L-LTP *in vitro* (Frey et al., 1988; Frey et al., 1993; Huang et al., 1994; Frey and Morris, 1997; Nguyen and Kandel, 1997; Frey and Morris, 1998a; Okulski et al., 2002; Scharf et al., 2002). In the experiments reported here, no effect of this drug at either 25 or 50 μ M was found on the magnitude or duration of the observed potentiation. Stanton and Sarvey, (1984) also reported that out of a number of different

protein synthesis inhibitors used to block L-LTP, anisomycin was also found not to produce the expected inhibitory effect. Others (Karl Bradshaw, pers. com.) have found similar results.

One possible explanation could be that in addition to anisomycin acting as a translational inhibitor, it also interferes with the regulation of a number of stress-activated MAP kinases, which may lead to the induction of several immediate early genes (Edwards and Machadevan, 1992; Hazzalin et al., 1998; Torocsik and Szeberenyi, 2000). Emetine and puromycin have been shown to produce negligible stress responses, whereas even concentrations of anisomycin that do not affect translation, can still cause gene induction (Edwards and Machadevan, 1992). Even if this were the case, whether such activation interacts with any of the processes triggered by the induction of LTP, leading to an enhancement of the response, is not entirely clear. Various procedures using either [^3H]-valine, (Stanton and Sarvey, 1984) [^3H]-leucine, (Deadwyler et al., 1987) or [^{35}S]-methionine, (Barea-Rodriguez et al., 2000) incorporation have shown that both *in vitro* and *in vivo*, anisomycin inhibits protein synthesis. However, inhibition may be required to reach a 'threshold' before effects on LTP are seen. Deadwyler et al., (1987), found that reduced but not complete LTP blockade was observed in slices when the level of protein synthesis inhibition was 79 % after a 15 min pre-incubation period. A 30 min pre-incubation period resulted in a level of inhibition of 85 % and this was found to completely block LTP. It is possible that protein synthesis was not blocked below this 'threshold' at the time of LTP induction, however this cannot be determined for certain. As this drug was ineffective, other experiments were conducted with the irreversible inhibitor emetine.

5.7.3 The effect of emetine on L-LTP.

Emetine has been previously been shown to block LTP in studies in which the potentiation was only followed for a relatively short period post-tetanus (Stanton and Sarvey, 1984). In the perforant pathway Nguyen and Kandel, (1996), found that L-LTP (recorded for 3 hr post-tetanus) was blocked by application of 25 μM emetine, when the drug was applied 10 min before ten high frequency trains, and was washed out 30 min later. The inhibitory effect of this drug was seen 80 min after the tetanus. An even earlier inhibitory effect, albeit with a higher concentration of emetine (100 μM) was reported by Huang et al., (2000). They recorded LTP for 3 hr in slices of amygdala, and found that LTP that persisted for the 3 hr recording period was inhibited after 1 hr by a 30 min pre-tetanus, (until 60 min post-tetanus) application of emetine.

An influential factor in the choice of a concentration of 100 μ M was that this concentration had been successfully shown to completely block L-LTP in mouse hippocampal slices by another research group. Karl Bradshaw (unpublished PhD thesis) was also using an identical recording chamber as the one used in the experiments reported here. However, a potentially important difference is that in the experiments conducted by Bradshaw, emetine was applied throughout the course of the experiment. Therefore, they may have achieved a more complete block of translation with such prolonged incubations. The present results with and [35 S]-methionine incorporation in slices incubated for 8 hr in emetine are consistent with this conclusion. Bradshaw did not test whether the drug applied post-tetanus had any effect on established LTP. This would control for effects on a possible constitutive depletion of proteins that may occur during long incubations in these inhibitors. The experiments reported in this chapter were conducted with 'brief' applications with a view to conduct further experiments on synaptic tagging. As an incomplete block of L-LTP was found, these planned experiments were not possible.

However, as the results from the experiments to measure the incorporation of [35 S]-methionine into hippocampal slices showed, when slices were incubated in the drug for similar durations as in the LTP experiments i.e., 1.5 hr protein translation was not blocked. This may have accounted for the modest effect of the drug in the LTP experiments. Even though basal translation of some protein was still occurring in the presence of emetine, it is likely that following a high frequency tetanus translation is massively upregulated. This would account for the partial effect that emetine had in comparison to the control experiments. Although emetine did not block translation in the untetanised slices in which the assay was conducted, it may have reduced levels of proteins in slices following a high frequency tetanus. Future experiments would be required to address these possibilities.

As a 1.5 hr incubation in 100 μ M emetine did not reduce the incorporation of [35 S]-methionine into slices, experiments were conducted where the slices were incubated for much longer periods. Longer incubation times may be required so the drug can completely penetrate the tissue and shut down translation throughout the slice. When slices were incubated in emetine for 8 hr, there was an almost complete inhibition of translation as indicated by the absence of almost any [35 S]-methionine label in this slice. It would have been advantageous to run a 'control' slice that was not treated with emetine, but incubated for 8 hr in order to confirm that this slice was still viable after 8 hr. Unfortunately, this was not possible as both slices were incubated in the same chamber at the same time. However, field EPSPs were

recorded from the 8 hr emetine treated slice so it was viable at the time of removal from the chamber.

Dr. J. U. Frey advised that long pre- experiment incubation times (up to 5 hr) are required to allow for any increase in translation following the preparation of slices to return to basal levels. In this preparation, fibres are severed and levels of glutamate release are increased. Slices may also become slightly hypoxic. It is known that during hypoxia and ischemia, there is a depolarisation of neurons that is associated with a change in their intracellular ionic composition (Hansen, 1985; Tanaka et al., 1997). Glutamate-induced excitotoxicity has been implicated as an important trigger of hypoxic damage (Choi, 1992). In a number of cell lines, it has been shown that hypoxia results in immediate early gene expression (Prabhakar et al., 1995). If in the present experiments, at the time of tetanus, the levels of proteins in these slices had been still high (due to activation at the time of preparation); a tetanus given during the presence of a drug like emetine may still have led to L-LTP. This may arise as ‘tagged’ synapses can subsequently capture the proteins that had been elevated at the time of tetanisation, thus giving rise to L-LTP. Incidentally, one group has reported that there is a dramatic and persistent inhibition of new protein synthesis in the CA1 neurons 2 h after 10 min hypoxia (Raley-Susman et al., 2001), so these concerns may not be a problem.

Again, a long pre-incubation of slices in protein synthesis inhibitors may allow for a clear dissection of these possibilities. Future experiments could be conducted where emetine would be applied for the entire pre-tetanus baseline period (possibly even before, i.e., during the pre-incubation phase). Baseline recordings would be conducted in the presence of the drug and (as in Bradshaw’s work) prior to tetanisation of one pathway. The drug could either be washed out after a certain period, or it may remain on the slice for the entire post-tetanus period.

5.7.4. The variable persistence of LTP.

The main result of the experiments reported here is that the persistence of LTP is not strictly determined by events at the time of the induction of LTP. E-LTP may be a first step in the process that can, depending on the ‘circumstances’, become a more enduring L-LTP. The circumstances include the prior and ‘future’ history of activation of the neurons such that they are tagged during the induction of E-LTP. If prior neuronal activity led to protein synthesis, then synapses stimulated at a level that induces only E-LTP may also display L-LTP. So

rather than has been suggested by Lanahan and Worley, (1998), “*macromolecular synthesis is required only during a brief time window immediately following a conditioning stimulus*”, (p.37), the persistence of LTP more likely results from the interaction of two dissociable events. The first involves local activation of specific synapses in a way that marks, or ‘tags’ them for a certain period. The second is the diffuse signalling to the cell body to synthesise proteins that upon distribution to the dendrites are captured by tags involved in the long-term changes defined as L-LTP.

Future experiments aimed at identifying the processes involved in the setting of synaptic tags would be beneficial. Persistently active kinases have been considered as candidate synaptic tags (Frey and Morris, 1998b; Martin and Kosik, 2002). Therefore, it would be interesting to see whether the heterosynaptic rescue of LTP is blocked, when ‘weak’ stimulation of one pathway in the presence of a kinase inhibitor, is followed by ‘strong’ stimulation to an overlapping pathway. This sort of experiment is somewhat complicated as some these molecules may also be involved in processes associated with the induction of LTP, independent of their involvement in setting of synaptic tags. The experiments so far have all used ‘weak’ stimulation that induces E-LTP, as a means of setting synaptic tags at the activated synapses. However, an interesting question would be to determine whether the setting of synaptic tags can be dissociated from the induction of LTP.

Another direction of investigation involves understanding the conditions that result in the triggering of protein synthesis. If heterosynaptic inputs are important in determining the variable persistence of LTP, it may be possible to demonstrate synaptic tagging and the paradoxical induction of L-LTP even when important heterosynaptic modulatory inputs to the hippocampus are blocked. The experiments reported in the next chapter focussed on the possible role that dopamine may play in triggering protein synthesis in the hippocampal slice.

Chapter 6. Dopamine and synaptic tagging.

6.1 Introduction.

Evidence suggests that activation of NMDA receptors alone is not sufficient to produce L-LTP, or that the persistence of LTP is not directly coupled to the degree of initial potentiation (Abraham et al., 1993). Dopamine was chosen as a likely candidate given that there is considerable evidence suggesting that the dopaminergic system plays an important role in modulating L-LTP (Frey et al., 1989a; Frey et al., 1990; Frey et al., 1991; Frey et al., 1993; Huang and Kandel, 1995). Two novel predictions were tested (1) Is it possible to rescue a 'normally' decremental form of LTP (in S2) by prior stimulation of an second input pathway (S1) with multiple high frequency tetani, when the NMDA receptor antagonist AP5 is present during the high frequency tetanus; and (2) If dopaminergic activation is necessary for L-LTP, it may be possible to induce dopamine-receptor dependent L-LTP even during the 'normal' blockade of L-LTP that would occur in the presence of a dopamine antagonist. This may be possible if, like the original synaptic tag experiment, high frequency stimulation of an overlapping independent pathway (S1) occurs prior to a second tetanus to an independent pathway (S2) in the presence of SCH23390.

6.1.1 The role of NMDA-receptor activation in L-LTP.

A number of 'possible' candidate synaptic tags were discussed in Chapter 1. Other than the possible identity of the synaptic tag(s), it is also unknown whether the tag is located pre- or postsynaptically. Much evidence in favour of a postsynaptic locus of expression of LTP was discussed earlier (see Section 1.9.4. of Chapter 1). (Collingridge et al., 1983) showed that the induction of LTP is blocked in the presence of the NMDA receptor antagonist AP5. Considerable evidence has accumulated that highlights how postsynaptic depolarisation of NMDA receptors is critical for LTP induction. The induction of L-LTP is also dependent on NMDA receptor activation (Reymann et al., 1989).

Preliminary unpublished data reported by Morris and Frey, (1999) suggest that setting of synaptic tags is also dependent upon NMDA receptor activation. In these experiments, one pathway (S1) was tetanised in the presence of AP5. Following washout of the drug, a second independent pathway (S2) was given a 'strong' tetanus that induced L-LTP on this pathway.

However, unlike the previous experiments discussed above, this time there was no rescue of the S1 pathway. The interpretation of these results is that as the NMDA receptor was blocked during the S1 tetanus and therefore these synapses were not tagged. The S2 tetanus resulted in L-LTP of this pathway, but had no effect on the S1 pathway, as no tags were present to utilise the proteins distributed in response to the 'strong' tetanus.

6.1.2 Dopamine and the hippocampus.

Neuroanatomical studies have shown that the hippocampus receives projections from the ventral tegmental area (VTA) and adjacent substantia-nigra (SN), (Carter and Fibiger, 1977; Swanson, 1982; Gasbarri et al., 1994a; Gasbarri et al., 1994b). Evidence suggests that this VTA projection to the hippocampus is dopaminergic. Verney et al., (1985) found that dopaminergic axons reached the hippocampal formation through the fimbria and the alveus, but also through the supracallosal bundle and the ventral amygdaloid area-entorhinal cortex. The temporal (ventral and caudal) part of the hippocampal formation received the bulk of the dopaminergic innervation. Further, they suggest that the ventral CA1 field appears to be the main target area for the hippocampal dopaminergic innervation. The dopamine D1 receptor is expressed more abundantly than the dopamine D2 receptor in the hippocampus and prefrontal cortex of non-human primates (Lidow et al., 1991) and rodents (Dubois et al., 1986). Immunohistochemical localisation of D1 and D5 receptors shows that there is heavy staining along pyramidal cells of the CA1 (Huang et al., 1992a), and retrograde tracers have shown that dopaminergic fibres from the ventral tegmental area (VTA) innervate the CA1 field (Verney et al., 1985; Gasbarri et al., 1994a).

6.1.3 Dopamine and LTP.

Many different transmitter systems appear to modulate synaptic plasticity, for example during cholinergically induced theta oscillation synaptic plasticity is greatly sensitised and can be induced by a single burst (4 pulses, 100 Hz). A burst given at the peak of theta induces homosynaptic LTP; the same burst at a trough induces homosynaptic LTD of previously potentiated synapses (Huerta and Lisman, 1993, 1995). In different subregions of the hippocampus various neuromodulators have been found effect LTP, such as β -adrenergic receptors (Dunwiddie et al., 1992), serotonergic receptors (Villani and Johnston, 1993). Dopamine has also been found to have a range of effects on synaptic plasticity in the hippocampus.

Dopamine (DA) D1/D5 receptor antagonists have been shown to slightly decrease the induction of LTP in area CA1, (Otmakhova and Lisman, 1996) but more dramatically, they prevent L-LTP from persisting more than a few hours, (Frey et al., 1989a; Frey et al., 1990; Frey et al., 1991) despite the fact that LTP is induced with ‘strong’ tetanisation protocols that should produce ample activation of NMDA receptors. Also, administration of D1/D5 agonists can induce a slow-onset potentiation, that has similar characteristics to the late, protein synthesis-dependent, phase of LTP (Huang and Kandel, 1995). D1/D5 receptors are coupled to adenylyl cyclase by the stimulatory G-protein G_s and produce a rise in cAMP (Kebabian and Calne, 1979). Matthies et al., (1997) found that mice lacking the dopamine D1 receptor subtype had deficits in the maintenance of L-LTP, and an associated complete loss of D1 receptors. Activation of D1/D5 dopamine receptors can also block depotentiation and this appears to be mediated by adenylyl cyclase and PKA, because it was mimicked by forskolin and inhibited by PKA inhibitors (Otmakhova and Lisman, 1998).

Much of the enzymatic machinery that is associated with dopaminergic target cells are present in the hippocampus. This includes dopamine uptake sites (Mennicken et al., 1992), DARPP-32 (Sakagami et al., 1994), and calmodulin-dependent phosphodiesterase PDE1B1 (Polli and Kincaid, 1994). *In vivo* studies have shown that dopamine modulates hippocampal acetylcholine release (Nilsson et al., 1992; Imperato et al., 1993; Hersi et al., 2000). In cultured hippocampal neurons, dopamine agonists have been shown to stimulate Ca^{2+} release from intracellular stores (Lezcano and Bergson, 2002).

Although setting of synaptic tags is likely to be dependent upon NMDA receptor activation Morris and Frey, (1999) establishing the conditions for L-LTP appears to involve other pathways in addition to glutamatergic activation of the NMDA receptor. Since pharmacological stimulation of the NMDA receptor alone, without the additional elevation of extracellular calcium does not produce L-LTP (Kauer et al., 1988a), the synergistic action of other inputs may be important. Application of cAMP and dopaminergic agonists have been shown to induce a slow-onset potentiation that is dependent on protein synthesis (Frey et al., 1993; Huang and Kandel, 1995).

Dopaminergic activation during ‘strong’ stimulation may be important in triggering the cascade leading to translation. The mechanism by which this might occur is unknown. The simplest possibility is that ‘strong’ stimulation of the Schaffer-collateral axons in CA1, also directly activates these dopaminergic axons. One must consider whether this is likely at least

in a hippocampal slice as preparation of the slices may cut all these axons. However, the terminals containing the transmitter may be present. Frey et al., (1990) has shown that after pre-loading hippocampal slices with [^{14}C] dopamine, high frequency stimulation was associated with an increase in release of the labelled transmitter, an effect not seen after low frequency test stimulation.

6.1.3 Heterosynaptic modulation of L-LTP by dopamine.

The experiments that were conducted in this chapter focussed on two main questions. If plasticity-proteins sequestered by the synapses of S2 are induced heterosynaptically, the expression of LTP on the 'strong' pathway (S1) may not be necessary for late-LTP to be induced on pathway S2. Impey et al., (1996) found that *lacZ* expression in CREB reporter mice was not blocked by AP5 following 'strong' tetanisation. Protein synthesis-dependent late-LTP may therefore be induced on S2 (despite LTP being absent on S1). Depending on the outcome of these experiments, future experiments may be possible; if AP5 during tetanisation of S1 were to be accompanied by a dopamine antagonist (e.g. SCH23390), late-LTP on S2 should be blocked if dopamine is the critical neuromodulatory transmitter.

Dopamine may play an important neuromodulatory role in L-LTP by triggering protein synthesis. Application of a dopamine receptor antagonist is predicted to block L-LTP with 'strong' stimulation. Having established this, synaptic tagging may take place in the presence of a dopamine antagonist. As with anisomycin (Frey and Morris, 1997), 'strong' stimulation to one pathway (S1) followed by a second 'strong' tetanus to an independent pathway (S2) but this time in the presence of a dopamine receptor antagonist may result in the paradoxical induction of L-LTP on S2. Although the dopamine antagonist may prevent protein synthesis the S2 synapses may be tagged and therefore sequester proteins originally synthesised in response to the S1 tetanus.

6.2 Methods.

6.2.1 Preparation of slices, and recording set-up.

Similar procedures for the preparation and incubation of slices were used as described Chapters 2 and 3.

6.2.2 Tetanus protocol.

‘Strong’ stimulation consisted of three high-frequency tetanic trains at 100 Hz in a 1 s burst, with an inter-train interval of 10 min (biphasic pulses with 200 μ s per half-wave i.e. twice the duration of test stimulation). In some experiments ‘strong’ tetanic stimulation was delivered to both pathways with an interval of 50 min between each tetanus. ‘Weak’ stimulation consisted of a single tetanus of 11 pulses at 100 Hz (biphasic pulses with 200 μ s per half-wave i.e. twice the duration of test stimulation), as in Chapter 4.

6.2.3 Preparation of drugs for electrophysiological experiments.

The NMDA receptor antagonist D-AP5 was prepared as a stock solution at 1 mM. The powdered drug was dissolved in equimolar NaOH and pH corrected to pH 7.4 with 1 M NaOH. The drug was dissolved in aCSF on each day of an experiment to a final concentration of 25 μ M.

For SCH23390, the powdered drug (19.9 mg) was dissolved in 20 ml of sterile, millipore filtered dH₂O to a concentration of 3 mM. Gentle sonication for ~ 10 min was required to completely dissolve the drug. On each day of an experiment the drug was dissolved in aCSF to a final concentration of 100 μ M. Both drugs were placed in a 50 ml beaker, wrapped in aluminium foil, and stored in a water bath to maintain the temperature of the solution at 32 °C throughout application. This solution was bubbled with 95:5 O₂/CO₂, throughout and the pH was found to be identical to the aCSF used to perfuse the slices i.e., 7.3 – 7.4. The drugs were applied to the slices by simply switching the tubing connected to the chamber between reservoirs. The perfusion flow rate was maintained at 150 μ l/min and drug application times were corrected for transit time from the reservoir to the slice (15 min).

6.4 Results.

Control experiments were conducted in order to verify that the induction of L-LTP could be blocked by delivering a high frequency tetanus in the presence of the NMDA receptor antagonist AP5 (25 μ M). Also, it was determined whether upon washout of AP5, L-LTP could be induced by high frequency stimulation of a second input pathway to the same group of cells. AP5 was applied 30 min before the tetanus to S1 and was washed out immediately after.

6.4.1 Experiments with AP5 during high frequency stimulation.

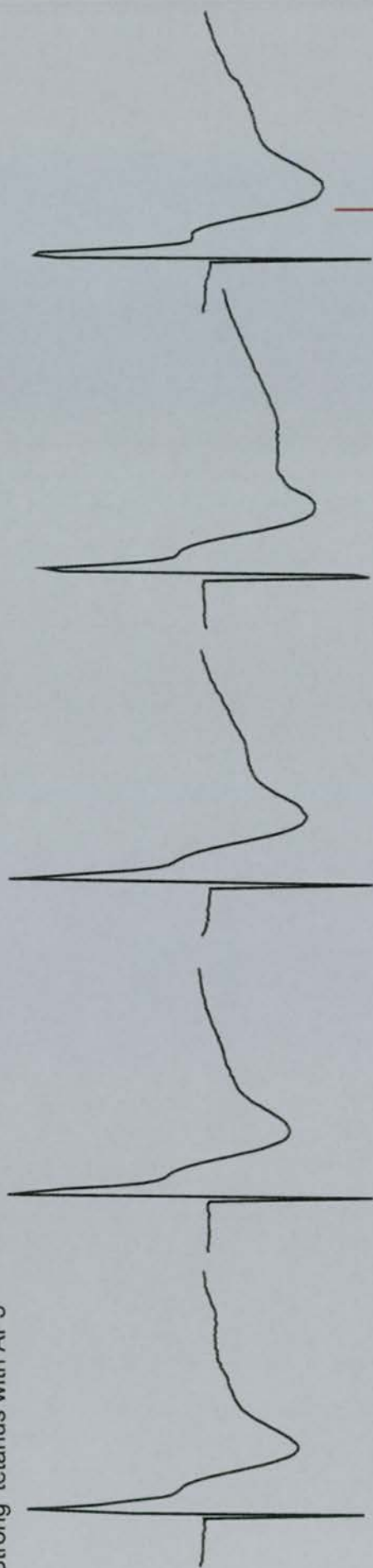
The averaged response over a range of stimulation intensities from 10 – 90 μ A increased from 0.74 to 3.71 mV/ms for S1, and from 1.31 to 3.83 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

Representative field EPSPs are shown (Figure 6.1) from an individual experiment where repeated ‘strong’ stimulation of one input pathway (S1) in the presence of 25 μ M AP5 was delivered to one pathway. Following washout of the drug, similar high frequency ‘strong’ stimulation was delivered to a second pathway (S2). There was no PTP in the S1 pathway after delivery of the first of three tetani to this pathway in the presence of AP5 (fEPSP Slope = $99.49\% \pm 2.38$ s.e.m., $t < 1$, $p > 0.84$). The control S2 pathway, also showed no PTP after the tetanus to S1 (fEPSP Slope = $105.89\% \pm 3.38$ s.e.m., $t = 1.75$, $p > 0.14$). Subsequent tetanisation of S2 resulted in significant PTP in this pathway when AP5 was no longer present (fEPSP Slope = $180.69\% \pm 11.70$ s.e.m., $t = 6.90$, $p < 0.005$).

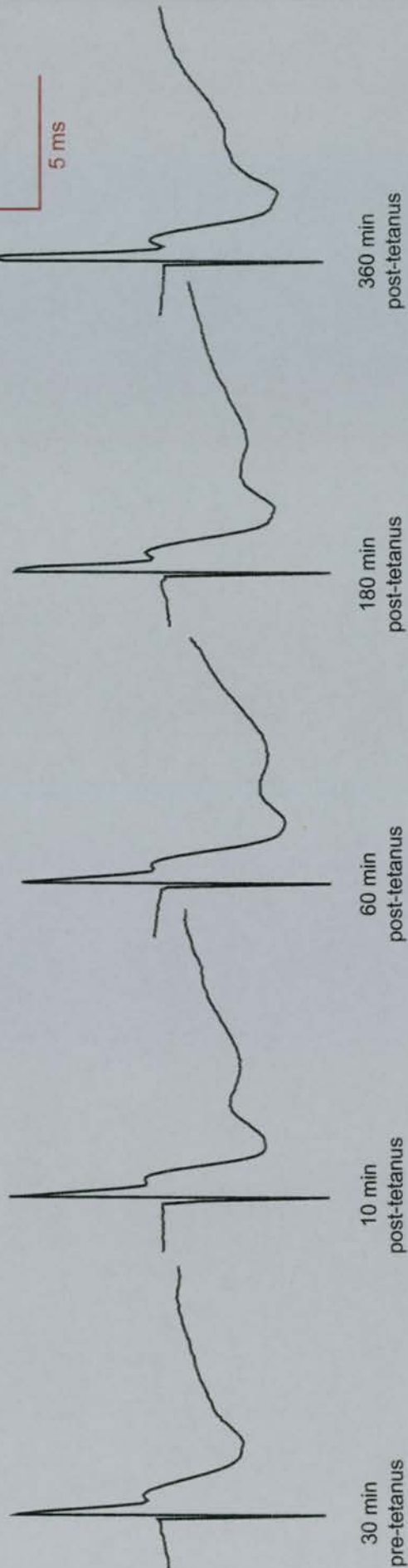
Figure 6.1 Representative fEPSP waveforms from an individual experiment where one pathway received ‘strong’ stimulation in the presence of 25 μ M AP5, following washout of the drug, the other pathway also received ‘strong’ stimulation.

Individual fEPSP's from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.2 a.

S1 - 'Strong' tetanus with AP5



S2 - 'Strong' tetanus without AP5



6.4.2 L-LTP is blocked by the NMDA receptor antagonist AP5.

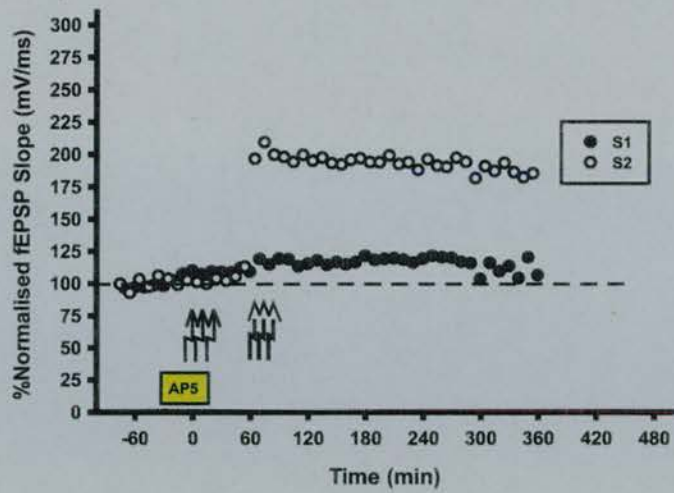
An individual example of an experiment where the S1 pathway was given ‘strong’ tetanic stimulation in the presence of AP5 (25 μ M) 1 hr before S2 received ‘strong’ stimulation (45 min after AP5 was washed out), is shown in Figure 6.2 a. Stable baseline responses were recorded for 1 hr before S1 was tetanised (‘strong’ stimulation). This ‘strong’ tetanus did not induce LTP, with the response being 106.89 % 10 min post-tetanus, and this remained at a stable level of 105.90 %, 360 min post-tetanus. After washout of the AP5 i.e., 1 hr later, S2 was given ‘Strong’ stimulation that resulted in an initial potentiation of 209.04%, 10 min post-tetanus, and this remained at an elevated level for the entire post-tetanus period (184.88% at 360 min).

The results of the experiments with AP5 present during high frequency stimulation of one input pathway (n=6) are shown in Figure 6.2 b. L-LTP was blocked in the presence of AP5, but could be induced in an independent pathway following wash out of the drug. A repeated measures ANOVA with pathway (with or without AP5) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors was conducted. Statistical analysis revealed that the pathway that was tetanised without AP5 (S2) did change significantly across time i.e., LTP was induced [$F(3, 15) = 3.91$; $p < 0.05$]. The presence of AP5 during a tetanus (S1) completely blocked LTP [$F(3, 15) = 1.55$; $p > 0.05$]. The averaged values \pm s.e.m. are presented in Table 6.3. Statistical comparisons (Paired samples t tests with Bonferroni correction for multiple comparisons) between the tetanus with and without AP5 (Figure 6.2 c) showed significant differences at all times post-tetanus.

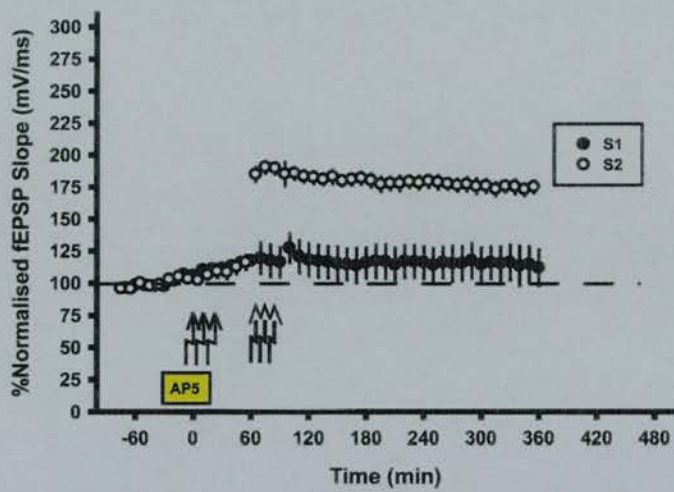
Figure 6.2 The induction of L-LTP is blocked by the NMDA receptor antagonist AP5.

- a. An individual experiment showing a representative example of the block of L-LTP with 'Strong' tetanisation to S1 in the presence of 25 μ M AP5, 60 min later a 'Strong' tetanus to a second input (S2) produces L-LTP on this pathway.
- b. Grouped data for 'Strong' before 'Strong' experiments with 25 μ M AP5 (n=6). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. 60min of pre-tetanus baseline response was recorded before three 100Hz tetanic trains (indicated by filled arrowhead) were delivered to S1 in the presence of AP5 (the drug was applied 30 min before the tetanus and washed out after the last tetanus, as shown on graph). 40 min after the last of the three trains (i.e., 60 min after the first train) three 100Hz tetanic trains were delivered to S2 (indicated by empty arrowhead) without AP5. Error bars indicate \pm s.e.m. Symbols: S1 strong tetanus pathway with AP5 ●. S2 strong tetanus pathway without AP5 ○.
- c. Statistical comparison between the S1 pathway which was tetanised in the presence of AP5, and S2 which was tetanised following washout of AP5. Paired samples t tests were conducted between the normalised responses measured in each pathway at a number of times post-tetanus. The values were taken at times relative to when each pathway received high frequency stimulation. (p values were adjusted according to Bonferroni correction for multiple comparisons).

a.



b.



c.

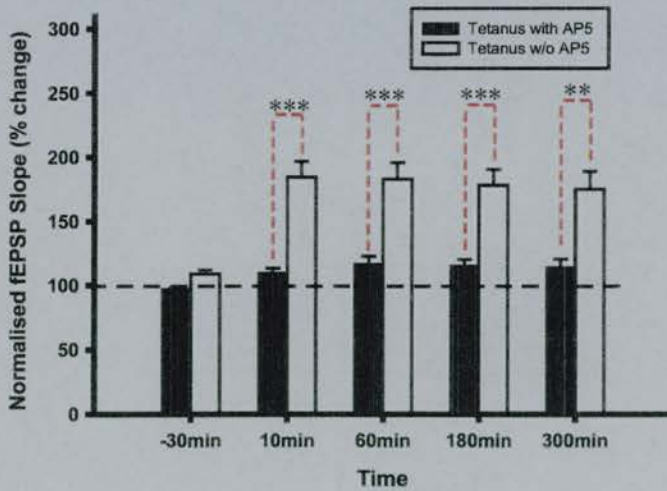


Table 6.3 Summary of statistical comparisons for AP5 ‘control’ experiments.

Time (min)	“Tetanus with AP5”, S1 pathway normalised EPSP slope	“Tetanus w/o AP5”, S2 pathway normalised EPSP slope	t value	
-30	97.47 ± 1.10	108.47 ± 2.87	2.88	p > 0.05
10	110.30 ± 3.23	190.51 ± 12.39	7.83	p < 0.005
60	117.30 ± 5.70	182.39 ± 12.43	6.73	p < 0.005
180	115.62 ± 4.81	179.73 ± 13.11	7.71	p < 0.005
360	111.86 ± 4.91	175.78 ± 15.07	5.58	p < 0.01

Table 6.3: Paired samples t-test comparisons of S1 pathway with AP5, to S2 pathway w/o AP5 at a number of times before and after either tetanus. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

6.5.1 Does ‘strong’ stimulation, delivered in the presence of an NMDA receptor antagonist, prolong LTP induced by subsequent ‘weak’ stimulation?

Experiments reported in Chapter 5 revealed that a ‘normally’ decremental LTP induced by ‘weak’ stimulation can be transformed into non-decremental LTP (or L-LTP) if prior to the ‘weak’ stimulus, ‘strong’ stimulation is delivered to an independent pathway to the same population of CA1 cells. The next series of experiments aimed at addressing whether or not ‘strong’ stimulation delivered 50 min prior to a ‘weak’ tetanus would result in L-LTP being induced in the ‘weak’ tetanus pathway, when the ‘strong’ tetanus was delivered in the presence of AP5. AP5 was applied 30 min before the tetanus to S1 and was washed out immediately after, i.e., 30min before the ‘weak’ tetanus to S2.

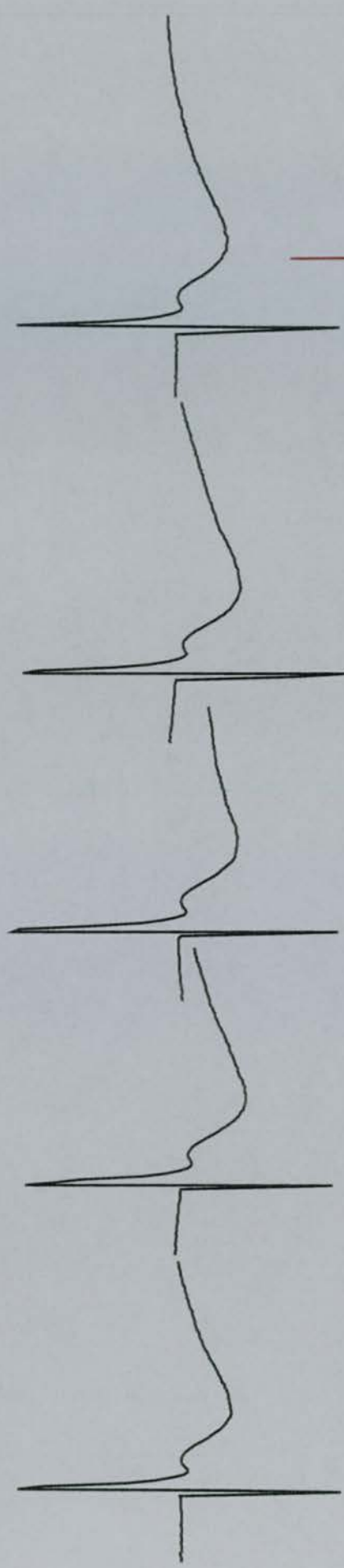
The averaged response over a range of stimulation intensities from 10 – 90 μ A increased from 0.54 to 2.90 mV/ms for S1, and from 0.14 to 3.28 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

Representative field EPSPs are shown (Figure 6.4) from an individual experiment in which repeated ‘strong’ stimulation of one input pathway (S1) in the presence of 25 μ M AP5. Following washout of the drug, a ‘weak’ tetanus was delivered to a second independent pathway (S2). There was no PTP in the S1 pathway after delivery of the first of three tetani to this pathway in the presence of AP5 (fEPSP Slope = $109.25\% \pm 6.22$ s.e.m., $t = 1.49$, $p > 0.1$). The S2 pathway, also showed no PTP after the tetanus to S1 (fEPSP Slope = $98.12\% \pm 3.64$ s.e.m., $t < 1$, $p > 0.6$). Subsequent ‘weak’ tetanisation of S2 resulted in significant PTP in this pathway when AP5 was no longer present (fEPSP Slope = $145.76\% \pm 6.32$ s.e.m., $t = 7.23$, $p < 0.005$).

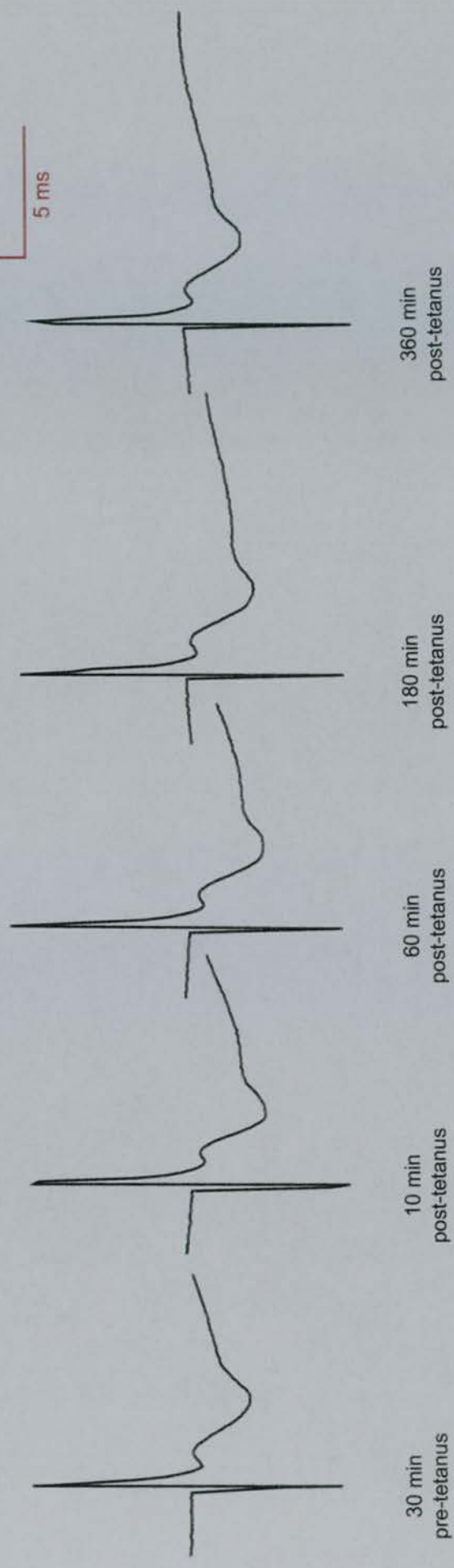
Figure 6.4 Representative fEPSP waveforms from an individual synaptic tag experiment with AP5 present during the ‘strong’ tetanus.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.5 a.

S1 - 'Strong' tetanus with AP5



S2 - 'Weak' tetanus



6.5.2 'Strong' stimulation in the presence of AP5 does not rescue decremental LTP induced by a 'weak' tetanus.

An individual example of an experiment where the S1 pathway was given 'strong' tetanic stimulation in the presence of AP5 (25 μ M) 50 min before S2 received 'weak' stimulation, is shown in Figure 6.5 a. Stable baseline responses were recorded for 1 hr before S1 was tetanised with AP5 ('strong' stimulation). This 'strong' tetanus did not induce LTP, with the response being 106.89 % 10 min post-tetanus, and this remained at a stable level of 105.90 %, 360 min post-tetanus. After washout of the AP5 i.e., 1 hr later, S2 was given 'weak' stimulation that resulted in an initial potentiation of 144.88 %, 10 min post-tetanus, however this LTP was not persistent and declined back to baseline levels 2 – 3 hr post-tetanus (reaching 98.05 % after 6 hr).

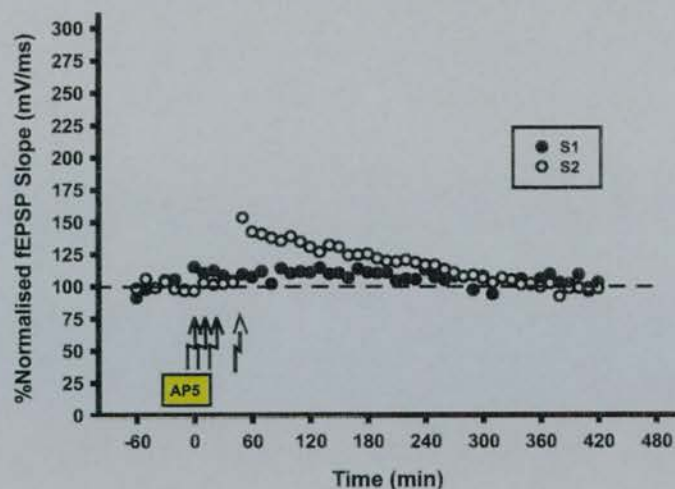
The results of the 'Strong before Weak', with AP5 present during the 'Strong' tetanus (n=6) are shown in Figure 6.5 b. In order to determine whether or not 'Strong' tetanisation (with AP5) had any effect on the duration of the potentiation induced with a subsequent 'Weak' tetanus, comparisons were made between these experiments, and experiments in which 'Weak' stimulation alone was delivered to just one pathway. A repeated measures ANOVA with time (10, 60, 180, 360 min) after the 'weak' tetanus as a within-subjects factor and experimental condition ('Weak' tetanus alone or 'Strong before Weak with AP5') as a between-subjects factor was conducted. There was no significant difference between the experiments [$F < 1$] indicating that a 'Strong' tetanus in the presence of AP5, delivered 50 min prior to a 'Weak' tetanus, did not affect the duration of the potentiation in the 'Weak' (S2) pathway. As was the case with 'Weak' stimulation alone, 'Strong before Weak, with AP5' resulted in decremental LTP.

Independent samples t tests showed that the S1 pathway from the 'Weak' alone experiments was not significantly different from the S1 pathway from the 'Strong before Weak with AP5' experiments at any time post-tetanus. These results are summarised in Table 6.6.

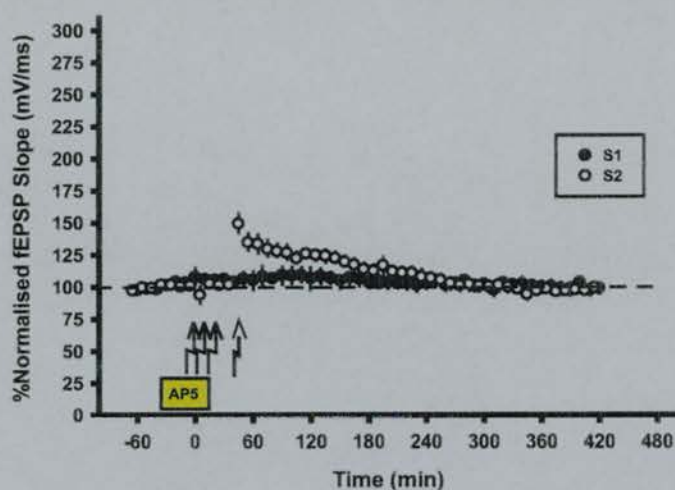
Figure 6.5 Synaptic tagging is dependent upon activation of the NMDA receptor during 'strong' high frequency stimulation.

- a. A representative example of the failure of 'strong' stimulation to rescue the potentiation induced with a 'weak' tetanus, when AP5 (25 μ M) is present during the 'strong' tetanus.
- b. Grouped data for 'Strong' before 'Weak' experiments with 25 μ M AP5 (n=6). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. 60min of pre-tetanus baseline response was recorded before three 100Hz tetanic trains (indicated by filled arrowhead) were delivered to S1 in the presence of AP5 (the drug was applied 30 min before the tetanus and washed out after the last tetanus, as shown on graph). 30 min after the last of the three trains (i.e., 50 min after the first train) a single tetanus of 11 pulses at 100 Hz was delivered to S2 (indicated by empty arrowhead). Error bars indicate \pm s.e.m. Symbols: S1 strong tetanus pathway ●. S2 weak tetanus pathway ○.
- c. Statistical analyses of the duration of LTP produced by a 'weak' tetanus after a 'strong' tetanus without AP5 compared to a 'weak' tetanus after a 'strong' tetanus with AP5. Heterosynaptic rescue of E-LTP induced by the 'weak' tetanus only occurred when the 'strong' tetanus was delivered without AP5 present. The times analysed are relative to when each individual pathway was stimulated. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

a.



b.



c.

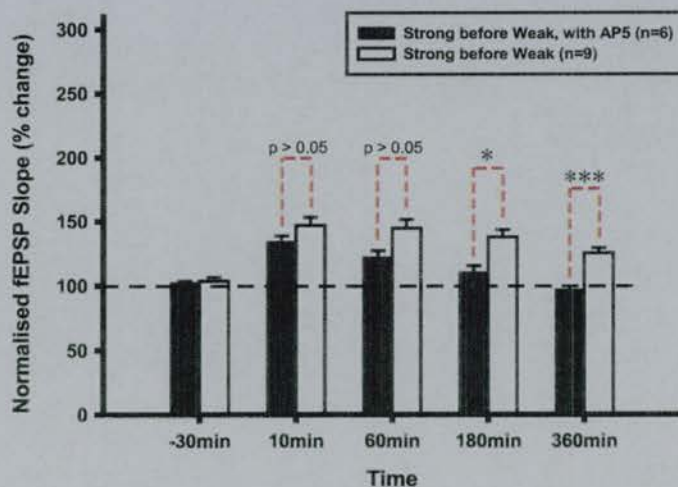


Table 6.6 Summary of statistical comparisons for synaptic tag experiments with AP5.

Time (min)	'Weak' alone normalised EPSP slope	'Weak' after 'Strong' with AP5 normalised EPSP slope	t value	
-30	102.74 ± 1.00	102.30 ± 1.22	< 1	p > 0.05
10	131.61 ± 5.32	134.40 ± 4.45	0.38	p > 0.05
60	119.64 ± 4.82	121.99 ± 5.16	0.33	p > 0.05
180	108.59 ± 3.61	110.49 ± 4.98	0.32	p > 0.05
360	92.38 ± 3.86	96.83 ± 2.85	0.87	p > 0.05

Table 6.6 Independent samples t-test comparisons of 'Weak' tetanus alone, with 'Weak' 50 min after a 'Strong' tetanus with AP5. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

6.6.1. Dopamine and L-LTP.

The first series of experiments assessed whether the selective D1 dopamine antagonist SCH23390 blocks L-LTP in the hippocampal slice. Previously, experiments by (Frey et al., 1991) used a 0.1 μM concentration of SCH23390 and observed a block of L-LTP. In a number of pilot experiments, L-LTP was not blocked with either 0.1 ($n=2$), 1 ($n=3$) or 10 ($n=2$) μM SCH23390. In order to get a significant block of L-LTP a concentration of 100 μM was necessary and this concentration was used in the experiments described below.

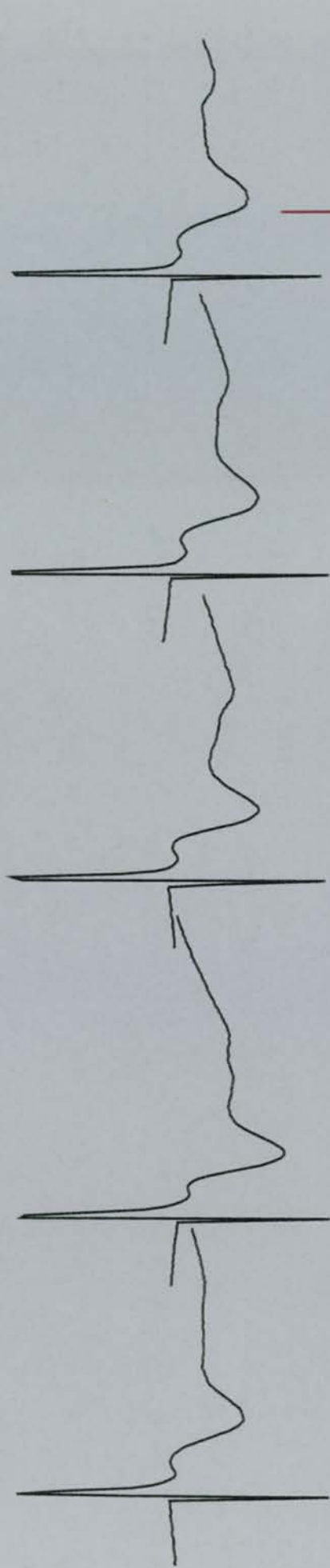
The averaged response over a range of stimulation intensities from 10 – 90 μA increased from 0.64 to 3.08 mV/ms for S1, and from 0.35 to 2.95 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

Representative fEPSPs are shown from individual experiments where SCH23390 was applied 30 min before a ‘strong’ high frequency tetanus was delivered to one pathway in Figure 6.7. There was a small change in the PTP measure of the S2 pathway after delivery of the first of three tetani to the S1 pathway (fEPSP Slope = $117.63\% \pm 2.69$ s.e.m., $t = 6.55$, $p < 0.005$) compared to the baseline level. This is due to the effect that application of the drug had on the baseline test responses before the S1 pathway was tetanised rather than a heterosynaptic interaction between the pathways. As can be seen from Figure 6.8 b, in these experiments the application of this drug to the slices resulted in a ‘drift’ in the basal test response by ~ 10 – 15% which reversed upon washout of the drug. The S1 pathway showed robust PTP after the ‘strong’ tetanus in the presence of SCH23390 (fEPSP Slope = $193.18\% \pm 7.99$ s.e.m., $t = 11.67$, $p < 0.005$).

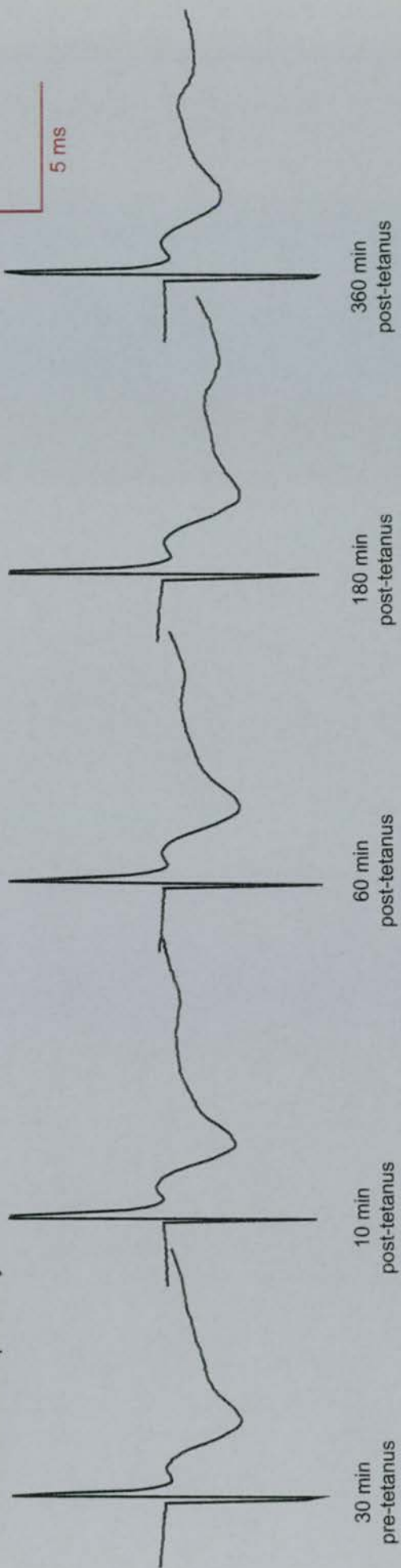
Figure 6.7 Representative fEPSP waveforms for an individual experiment where SCH23390 (100 μ M) was applied during a ‘strong’ high frequency tetanus.

Individual fEPSPs from both the tetanus (S1) and the non-tetanus control pathway (S2) recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.8 a.

S1 - Tetanised pathway



S2 - Non-tetanised pathway



5 mV

5 ms

360 min
post-tetanus

180 min
post-tetanus

60 min
post-tetanus

10 min
post-tetanus

30 min
pre-tetanus

6.6.2 Block of L-LTP with the dopamine antagonist SCH23390 (100 μ M).

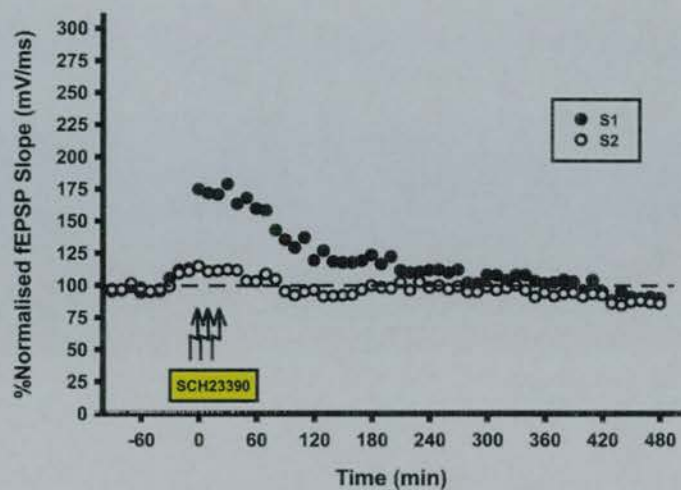
An individual example of an experiment where SCH23390 (100 μ M) was applied 30 min before and washed out 60 min after, a ‘strong’ high frequency tetanus was delivered to one pathway (S1) is shown in Figure 6.8 a. Initially the level of potentiation was 171.29 % at 10 min post-tetanus, however this LTP was decremental and had declined to a level of 122.93 %, 180 min post-tetanus, returning to a baseline level of 100.78% at 360 min post-tetanus with no significant change in the control pathway S2.

The results of experiments with 100 μ M SCH23390 (n=8) are shown in Figure 6.8 b. The presence of the D1 dopamine receptor antagonist SCH23390, during a high frequency tetanus completely blocked L-LTP with no effects on an independent control pathway for up to 8 hr post-tetanus. Statistical analysis confirms this conclusion. A repeated measures ANOVA with pathway (S1 and S2) and time (10 min, 60 min, 180 min, 360 min) as within-subjects factors was conducted. There was a significant difference between the pathways [$F(1, 7) = 94.31$; $p < 0.005$], indicating that the tetanus initially produced robust LTP. A significant pathway \times time interaction was found [$F(3, 21) = 28.78$; $p < 0.005$]. Subsequent analysis revealed a main effect of time in the tetanus pathway (S1) [$F(3, 21) = 55.88$; $p < 0.005$], reflecting the fact that the LTP was decremental. There was also a main effect of time for the control pathway (S2) across these intervals [$F(3, 21) = 25.18$; $p < 0.005$], reflecting the fact that the basal response changed as a result of application, and subsequent washing off, of the drug discussed earlier. The decremental nature of the potentiation induced in the presence of SCH23390 was confirmed by subsequent statistical analysis. The averaged values \pm s.e.m. are presented in Table 6.9. Statistical comparisons (Paired samples t tests with Bonferroni correction for multiple comparisons), between the tetanised and the control pathways showed no differences during E-LTP (up to 180 min post-tetanus), but L-LTP (measured at 360 min post-tetanus) was clearly effected (Figure 6.8 c.). Interleaved control experiments were performed whereby no drug was applied to slices and similar tetanus protocols were followed. The results of these experiments (n=3) are shown in Figure 6.8 d. These experiments clearly indicated that robust L-LTP was induced in the absence of SCH23390, see Figure 6.8.e for a comparison of both groups of experiments. The duration and magnitude of the LTP induced in these interleaved ‘control’ experiments was similar to that reported in identical experiments in Chapter 3, enabling statistical comparisons to be made between L-LTP in the ‘controls’ without drug, and when the drug was present. These results are depicted in Figure 6.8 f.

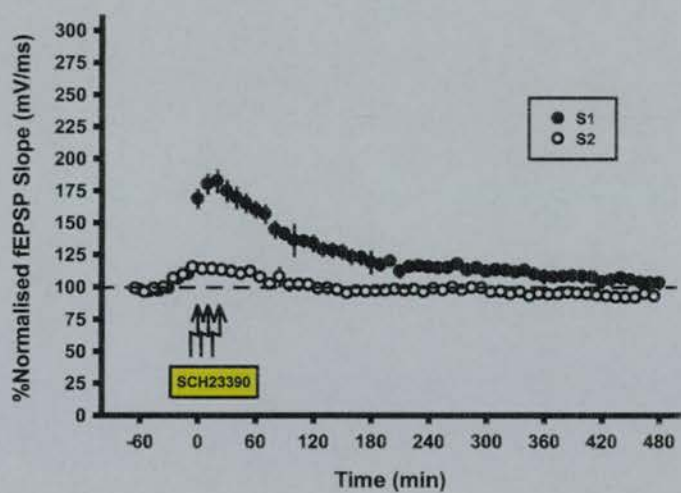
Figure 6.8 L-LTP is blocked by the D1-dopamine receptor antagonist SCH23390 (100 μ M).

- a. An individual experiment showing a representative example of the block of L-LTP in the presence of SCH23390 (100 μ M).
- b. Grouped data for experiments where SCH23390 (100 μ M) was applied 30 min pre-tetanus until 60 min post-tetanus (n=8). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. Error bars indicate \pm s.e.m. Symbols: S1 tetanus pathway ●. S2 non-tetanus control pathway ○.
- c. Statistical analyses of the duration of LTP produced by high frequency stimulation in the presence of the dopamine antagonist SCH23390. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at each time point. *** indicates a statistically significant difference with $P < 0.005$, ** with $P < 0.01$, * with $P < 0.05$ (Paired Students t-test, with Bonferroni correction for multiple comparisons). Error bars indicate \pm s.e.m. (n=8).
- d. Results of interleaved control experiments where high frequency stimulation was delivered without any drug present. In these experiments (n=3) robust, non-decremental L-LTP was induced. S1 tetanus pathway ●. S2 non-tetanus control pathway ○.
- e. Comparison of the experiments with and without SCH23390 (100 μ M) showing the S1 tetanus paths alone. Error bars indicate \pm s.e.m. Symbols: ● = tetanus with SCH23390; ○ = tetanus without SCH23390.
- f. Statistical comparison of L-LTP with and without SCH23390 (100 μ M). Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at each time point. *** indicates a statistically significant difference with $P < 0.005$, ** with $P < 0.01$, * with $P < 0.05$ (Paired Students t-test, with Bonferroni correction for multiple comparisons). Error bars indicate \pm s.e.m.

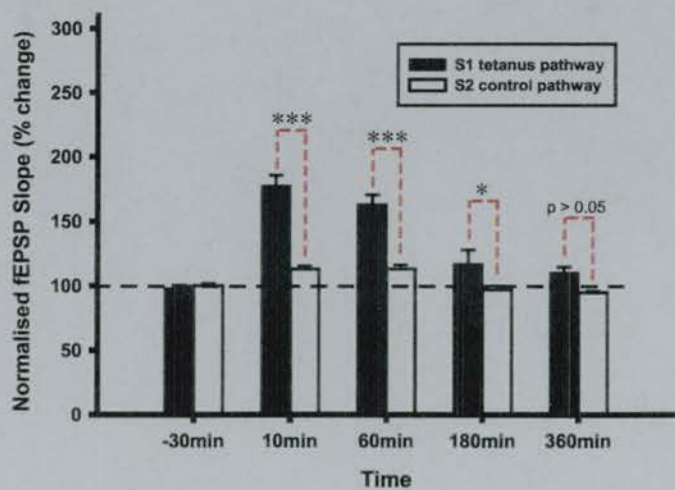
a.



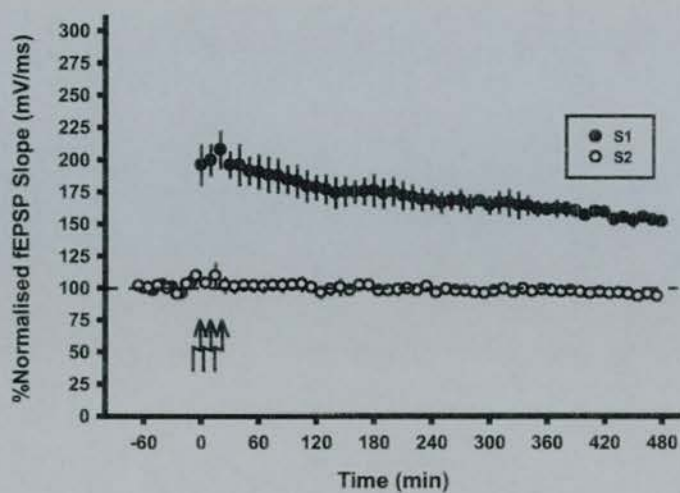
b.



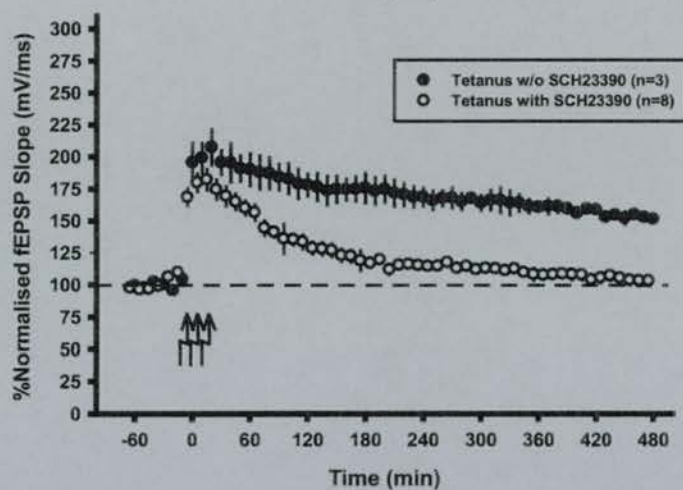
c.



d.



e.



f.

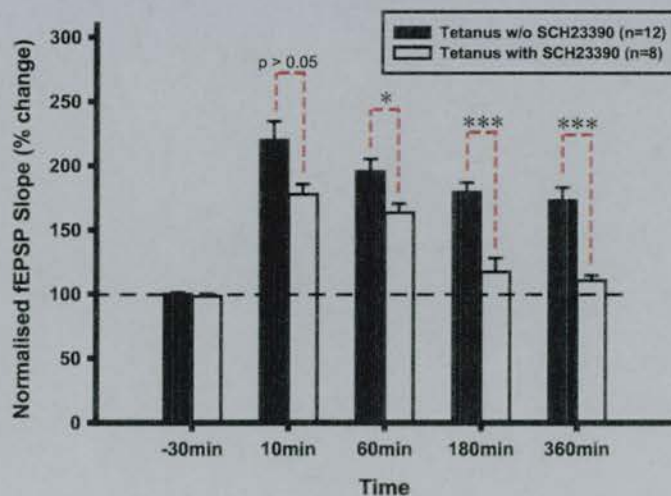


Table 6.9 Summary of statistical comparisons for experiments with SCH23390 (100 μ M).

Time (min)	S1 tetanus pathway normalised EPSP slope	S2 control pathway normalised EPSP slope	t value	
-30	98.94 \pm 1.50	99.90 \pm 1.22	< 1	p > 0.68
10	179.90 \pm 7.00	113.85 \pm 1.73	10.22	p < 0.005
60	160.04 \pm 5.65	111.81 \pm 2.52	9.77	p < 0.005
180	122.75 \pm 4.36	96.38 \pm 2.62	4.57	p < 0.05
360	108.19 \pm 4.65	94.70 \pm 0.94	3.02	p > 0.05

Table 6.9: Paired samples t-test comparisons of S1 pathway and S2 pathway at a number of times before and after either tetanus with SCH23390. For all analyses, the p values were adjusted according to Bonferroni correction for multiple comparisons.

6.7.1 Synaptic tagging and dopamine.

Synaptic tagging experiments were conducted where one pathway (S1) received 'strong' stimulation, 10 min after this, SCH23390 was bath applied and left to incubate for 30 min prior to a second 'strong' tetanus to a second independent input (S2). The drug was washed out 60 min after the tetanus to S2.

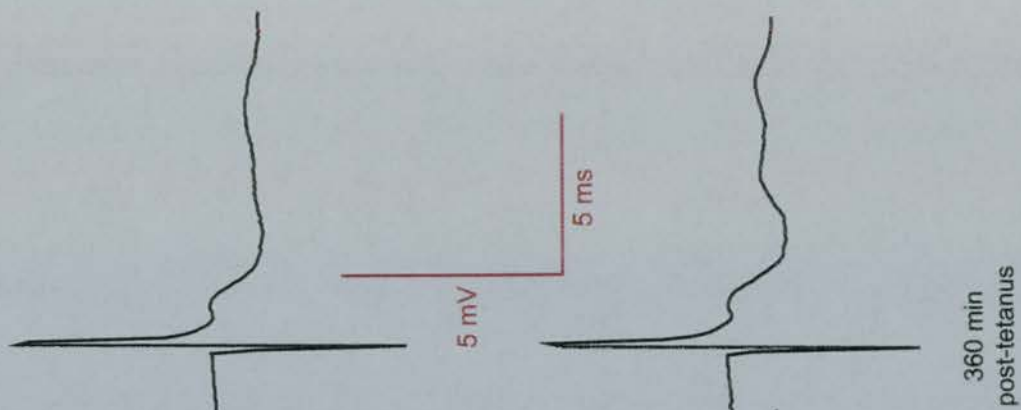
The averaged response over a range of stimulation intensities from 10 – 90 μ A increased from 0.82 to 3.11 mV/ms for S1, and from 0.24 to 3.21 mV/ms for S2 with no difference between pathways in the response to stimulation across all intensities [$F < 1$].

Representative fEPSPs are shown from an individual SCH23390 synaptic tag experiment in Figure 6.10. The times and waveforms are taken relative to when each pathway was tetanised. There was no PTP in the S2 pathway after delivery of the first of three tetani to the S1 pathway (fEPSP Slope = $104.25\% \pm 3.10$ s.e.m., $t = 1.37$, $p > 0.05$) compared to the baseline level. The tetanus pathway (S1) showed robust PTP (fEPSP Slope = $177.24\% \pm 6.72$ s.e.m., $t = 11.49$, $p < 0.005$). When a subsequent 'strong' tetanus was delivered to the S2 pathway this time in the presence of SCH23390, robust PTP was observed (fEPSP Slope = $175.59\% \pm 8.75$ s.e.m., $t = 8.64$, $p < 0.005$).

Figure 6.10 Representative fEPSP waveforms for an individual SCH23390 synaptic tag experiment.

Individual fEPSP's from both the tetanus (S1) without SCH23390 present at the time of stimulation, and the second tetanus pathway (S2), when SCH23390 was present, recorded from stratum radiatum of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.11 a.

S1 - 'Strong' tetanus without SCH23390 (100 μ M)

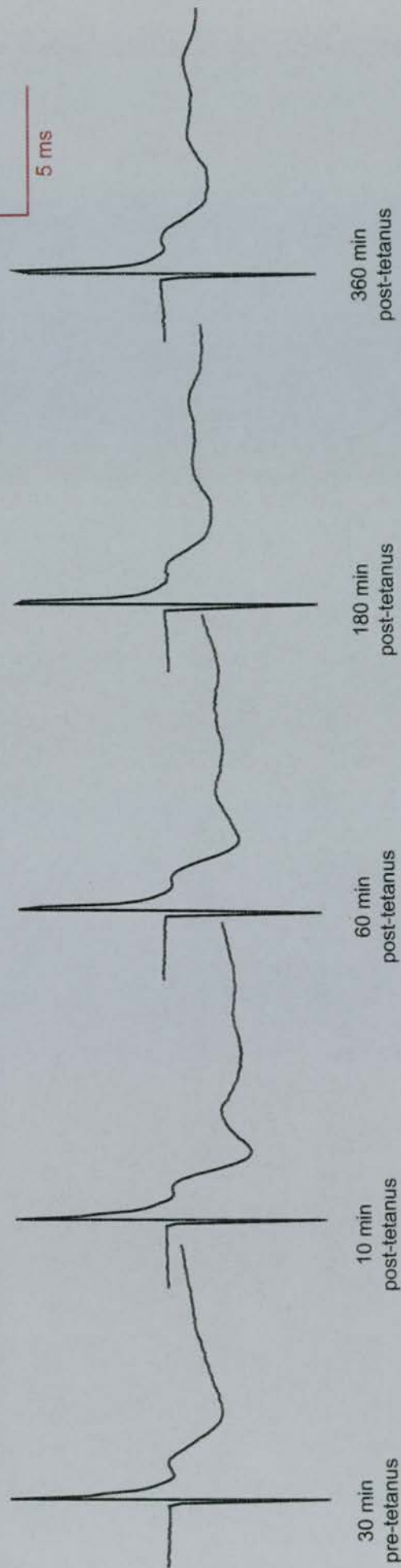


360 min
post-tetanus

180 min
post-tetanus

60 min
post-tetanus

S1 - 'Strong' tetanus with SCH23390 (100 μ M)



30 min
pre-tetanus

10 min
post-tetanus

60 min
post-tetanus

180 min
post-tetanus

360 min
post-tetanus

6.7.2 The absence of 'synaptic tagging' with the dopamine antagonist SCH23390.

An example of an individual experiment is shown in Figure 6.11 a. The 'strong' tetanus to S1 induced robust LTP, with the level of potentiation being 195.46 % 10 min post-tetanus, however this LTP was decremental and had declined to a level of 108.80 %, 180 min post-tetanus. When S2 was given a 'strong' tetanus, this time in the presence of SCH23390, the initial level of potentiation was 210.09 % 10 min post-tetanus, however the LTP in this pathway was also decremental and had declined to 106.14 % at 230 min post-tetanus (i.e., 180 min post-tetanus to S2).

The key finding of these synaptic tag experiments with SCH23390 (n=8) is shown in Figure 6.11 b. The results of these experiments revealed that a post-tetanus infusion of the D1 receptor antagonist SCH23390 inhibited established LTP in the S1 pathway, and also prevented the maintenance of LTP in the S2 pathway. Statistical comparisons of the SCH23390 synaptic tag experiments to controls (where no drug was applied), confirmed that SCH23390 reversed established LTP was in the S1 pathway [$F(1, 18) = 19.5$, $p < 0.005$], and also prevented the maintenance of LTP in S2 when the drug was present during this tetanus [$F(1, 18) = 17.6$, $p < 0.005$]. This effect is unlikely to be due to a general decline in the response due to poor slice health, as both pathways returned to the pre-tetanus baseline level and remained stable at this level for up to 8 hr post-tetanus. An interleaved control experiment clearly demonstrated that L-LTP could be readily induced without the drug present (see Figure 6.11 c).

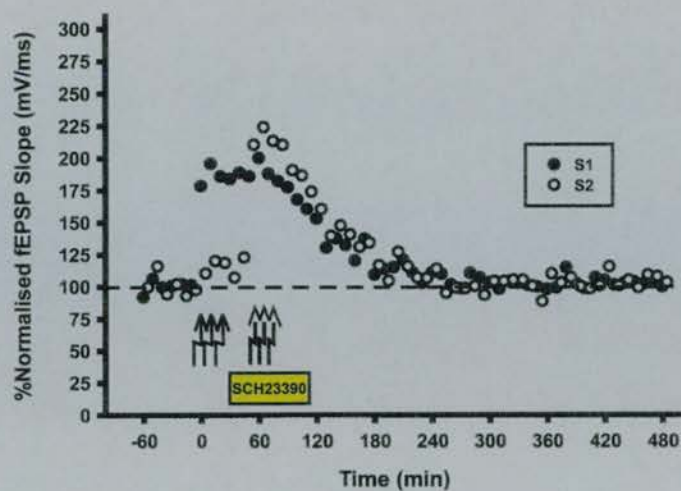
Independent sample t-tests showed that both the S1 and S2 pathways from the 'SCH23390 synaptic tag' experiments were significantly different from control experiments where the drug was not present this can be clearly seen in Figure 6.11 d.

The effect that SCH23390 had on LTP in S1 was unexpected. An individual experiment was subsequently conducted where one pathway (S1) received 'strong' stimulation and SCH23390 was applied 10 min post-tetanus, and perfused for 90 min in total as in the previous experiments. The initial level of potentiation was 200.55 % at 10 min post-tetanus, however this LTP was decremental and had declined to 153.30 % at 180 min post-tetanus, remaining at 127.16 % 10 hr after the tetanus with no change in the control S2 pathway.

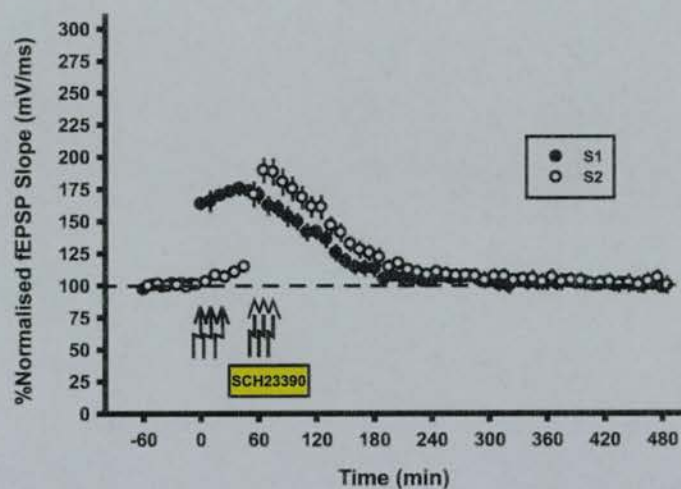
Figure 6.11 The absence of synaptic tagging in the presence of SCH23390.

- a. An individual experiment showing a representative example of the absence of synaptic tagging in the presence of SCH23390 (100 μ M).
- b. Grouped data for experiments where SCH23390 (100 μ M) was applied 30 min pre-tetanus until 60 min post-tetanus (n=8). Field EPSP slope, expressed as the percentage change normalised to the pre-tetanus baseline level is shown. Baseline (one hundred percent) was taken as the mean of the values in consecutive 10min periods (i.e. 5 successive evoked responses), and 60min of pre-tetanus baseline response was recorded before three 100Hz tetanic trains (indicated by arrows) were delivered to S1. Error bars indicate \pm s.e.m. Symbols: S1 tetanus pathway ●. S2 non-tetanus control pathway ○.
- c. A control experiment was conducted during the experiments with SCH23390 to insure that there were no problems recording LTP for long-periods at this time. This interleaved experiment clearly demonstrates that non-decremental homosynaptic L-LTP was induced by high frequency stimulation of S1.
- d. Comparison of LTP without SCH23390 to that induced in the synaptic tag experiments. LTP in the S1 pathway (i.e., SCH23390 post-tetanus) and the S2 pathway from the synaptic tag experiment (i.e., SCH23390 during tetanus) was clearly decremental in comparison to the 'control' experiments with no drug.
- e. An individual experiment (n=1) where 'strong' high frequency stimulation was delivered to S1. 10 min after the last tetanic train SCH23390 (100 μ M) was bath applied. L-LTP was blocked by post-tetanus application of SCH23390. The duration of drug application was similar to that in the 'synaptic tag' experiments above.

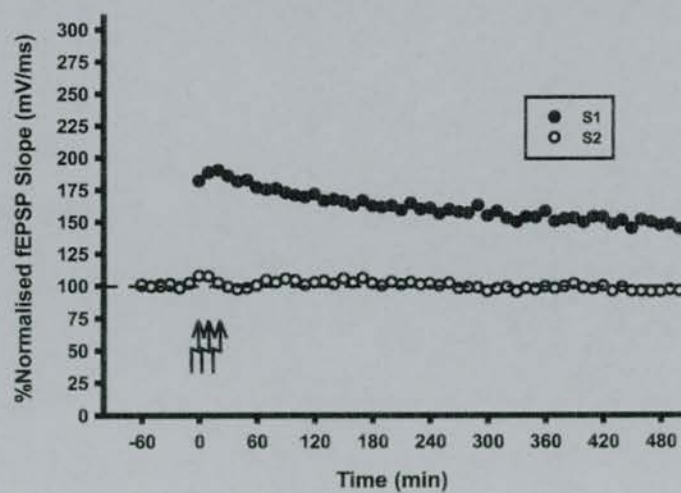
a.



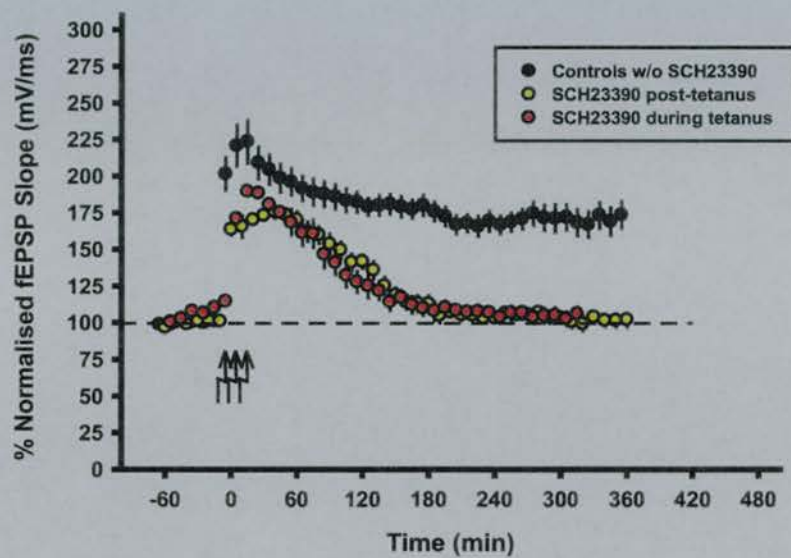
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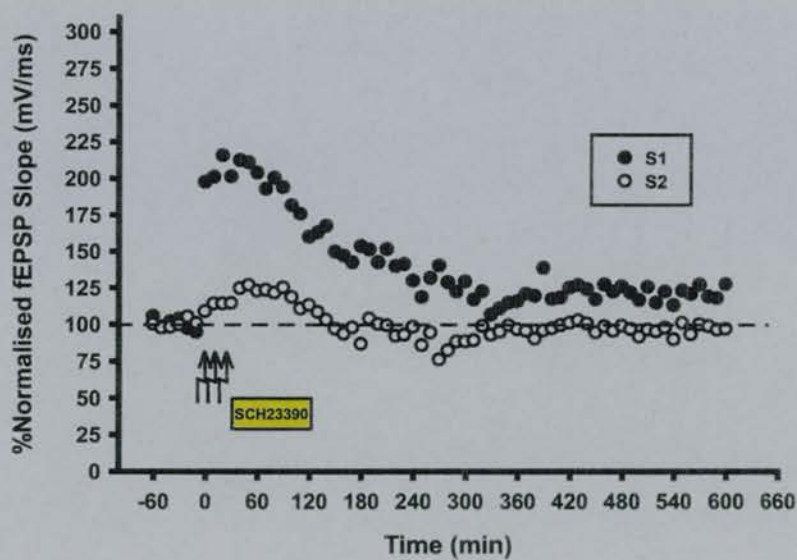
c.



d.



e.



6.4 Discussion.

6.4.1 Synaptic tagging is dependent upon activation of glutamate receptors.

‘Strong’ stimulation in the presence of AP5 was hypothesised to trigger the synthesis of proteins, possibly by dopamine release during the tetanus. Even though the NMDA receptor would be blocked during the high frequency tetanus, a situation leading to the failure to induce LTP (Collingridge et al., 1983), glutamate would still be released from the presynaptic cells. This glutamate may have ‘primed’ the subsequent induction of LTP with a ‘weak’ tetanus. Raymond et al., (2000) found that the induction of LTP was completely blocked by the application of AP5 during a theta burst tetanus. Subsequent ‘weak’ stimulation of the same pathway resulted in facilitated LTP compared to that induced without the ‘priming’ stimulus i.e., theta burst stimulation in the presence of AP5.

There are a number of possible reasons why L-LTP failed to be induced in the synaptic tag experiments with AP5. Dopamine release has been found to occur following high frequency stimulation (Frey et al., 1990). Although no direct measurement of dopamine release was made in these experiments, the experiments with SCH23390 confirmed that dopamine receptor blockade inhibited the maintenance of LTP for more than 2–3 hr post-tetanus. If dopamine release occurred during the ‘priming’ stimulation of S1 in the presence of AP5 it is possible that as the NMDA receptor was blocked at this time, and sufficient levels of intracellular Ca^{2+} were not reached in order to activate the signalling pathways leading to the protein synthesis cascade. Synergistic activity between both NMDA and dopaminergic receptors may be required for the synthesis and distribution of the ‘plasticity-proteins’ throughout the postsynaptic neuron.

As mentioned above, experiments by Raymond et al., (2000) used synaptic stimulation (two theta burst trains) in the presence of AP5 to ‘prime’ the subsequent induction of LTP by weak stimulation. This effect was mediated by group 1 mGluRs and was dependent on protein synthesis triggered by the priming event. The authors noted that in these synaptic priming experiments there was a stronger facilitation of LTP at early time points after the induction of LTP than that observed when the priming was induced by pharmacological activation with the mGluR agonist DHPG. They suggest that “*This may reflect the release of other neurotransmitters in addition to glutamate, such as noradrenaline, which is capable of*

priming the initial LTP induction but not its persistence” (p. 971). Release of other neuromodulators was suggested to provide an explanation for the residual early facilitation in slices treated with a group 1 mGluR antagonist. However, there have been no studies reported in the literature of dopaminergic activation being able to heterosynaptically rescue decremental LTP.

Raymond et al., (2000) reported that synaptic stimulation of one pathway in the presence of AP5 was not associated with priming of subsequent LTP induction on an independent pathway given a weak tetanus. In the synaptic tag experiments of Frey and Morris, (1997, 1998a), proteins are said to be transported to all synapses, thereby enabling the heterosynaptic induction of L-LTP when protein synthesis is blocked due to the setting of tags at the activated synapses. The situation may be different for mGluR priming of LTP. Whether dopamine regulates the persistence of L-LTP in an analogous way by triggering local dendritic protein synthesis, such that these proteins are restricted to the primed synapses, is not known at present. At a recent scientific meeting Smith et al., (2002) presented data using local perfusion of the D1 agonist SKF38393 to small regions (5 - 15 μm) of the distal dendritic arbor of cultured hippocampal neurons. Their preliminary results indicate that focal application of the agonist results in increased protein synthesis (measured by green fluorescent protein expression) that is often restricted to the area of the agonist perfusion. If dopamine triggers synapse-specific local translation, then this could explain the failure to observe a heterosynaptic rescue in the present experiments.

Future experiments could address some of these possibilities. The synaptic tag hypothesis predicts that heterosynaptic interactions are possible, and indeed do occur. We hypothesised that the ‘weak’ heterosynaptic activation may have tagged the S2 synapses in a way that they were able to capture proteins synthesised by the S1 tetanus, even though the NMDA receptor was blocked at this time. If ‘priming’ the synapses in the presence of AP5 somehow restricts the effect to the same synapses active during both the ‘priming’ and subsequent ‘weak’ stimulation, the following experiment may reveal L-LTP in the ‘weak’ stimulated pathway. As before, recordings would be made from two independent pathways. One pathway, S1, would receive ‘strong’ stimulation in the presence of AP5; after wash out of the drug, the same pathway S1 would subsequently be given ‘weak’ stimulation, with S2 acting as an independent control throughout. If proteins are distributed homosynaptically, then the potentiation on S1 would be non-decremental, i.e., L-LTP following a ‘weak’ tetanus that normally leads to E-LTP.

However, there are some potentially important differences between the experiments conducted and those of Raymond et al., (2000). The experiments of Raymond et al., (2000) were not concerned with differentiating between E-LTP and L-LTP, and indeed their recordings lasted for only 1 hr post-tetanus. Therefore, whereas their experiments concern the 'priming' of E-LTP, our experiments were concerned with the ability of priming to modulate L-LTP. Raymond et al., (2000) used a 'priming' stimulus (i.e., two theta burst trains) that they showed was dependent upon activation of group 1 mGluRs, as it was blocked by the group 1 antagonist AIDA. The 'priming' stimulus used in the present experiments was a repeated high frequency tetanus, which may activate different signalling pathways, therefore these two experiments may involve different mechanisms. It would also be advantageous to demonstrate that dopamine is released by high frequency stimulation, and that this also occurs in the presence of AP5.

6.4.2 Post-tetanus application of a dopamine antagonist reverses LTP.

It was surprising to find that SCH23390 blocked L-LTP when applied both before and after a high frequency tetanus. The mechanism of this effect is not entirely clear and future work would be required to characterise this further. The effect that dopamine has on LTP is mediated by the production of cAMP and the subsequent activation of PKA. Application of the PKA inhibitor KT 5720, 15 min after a tetanus had no effect on the level of the established potentiation (Huang and Kandel, 1994). Experiments by Frey et al., (1991) found that application of SCH23390 immediately after high frequency stimulation had no effect on LTP recorded for 3 hr post-tetanus. There are some reports indicating that SCH23390 may also act as both an antagonist (Hicks et al., 1984; Bischoff et al., 1986; Bischoff et al., 1988; McQuade et al., 1988; Bijak and Smiaowski, 1989), and as an agonist of 5-HT receptors (Millan et al., 2001). In the nucleus accumbens, SCH23390 has even been reported to have D1 receptor agonist properties (Wachtel and White, 1995). If SCH23390 also activated 5-HT receptors then this may explain why an inhibitory effect of the drug was found when perfused onto slices post-tetanus. A number of studies have found that activation of 5-HT receptors blocks the induction of LTP in the hippocampus (Villani and Johnston, 1993; Maeda et al., 1994; Edagawa et al., 1998).

It seems unlikely that the antagonist had any 'side-effects' on hippocampal slices such that the post-tetanus effect resulted from an effect on slice health and not LTP *per se*. In the synaptic tag experiments with SCH23390 presented in this chapter, the response of the S1

pathway was found to decline to the pre-tetanus baseline level and did not deteriorate below this level for up to 8 hr post-tetanus. Also, in the 'control' series of experiments where the drug was applied before, and during a 'strong' tetanus to one pathway, no effect on the S2 control pathway were observed, which would suggest that the effect that the antagonist has on inhibiting LTP is due its effects activated by the tetanus or shortly after, and not as a result of some non-specific effects of the drug. An individual experiment was conducted with a post-tetanus application of the antagonist after 'strong' stimulation of just one pathway. This resulted in a similar 'reversal' of established LTP with the S2 control pathway remaining stable for the entire post-tetanus period. Future experiments with longer delays between the tetanus and drug application would establish the temporal dynamics of post-tetanus dopaminergic activation in the maintenance of LTP.

Reducing the dose of the drug may also provide a way of resolving these questions. Whereas Frey et al., (1991) used SCH23390 at 0.1 μM and successfully blocked L-LTP, no effect on LTP was observed in pilot experiments using this low concentration or higher (1 and 10 μM , data not shown). Initial experiments with a dose of 100 μM demonstrated the effectiveness of this concentration on L-LTP with no effects on a simultaneously monitored control pathway. By either reducing the dose of the drug or applying it for shorter time periods before and during the tetanus future experiments may clear up these discrepancies. Lower doses or shorter applications may still result in a block of LTP during a tetanus, but without the post-tetanus reversal of LTP. Given more time, future experiments of this kind would enable synaptic tagging experiments to be replicated according to the same procedure as described here.

6.4.3 The role of dopamine in L-LTP.

The results of the synaptic tag experiments with SCH23390 led to the suggestion that dopamine release after a tetanus is important for the maintenance of LTP. This would be possible if some 'tonic' release of dopamine occurs in the hippocampal slice following high frequency stimulation. Alternatively, there may be a sustained period of release (at least for 10 min following a tetanus according to the present experiments) of dopamine that is important in triggering L-LTP. There have been no reports of 'tonic' dopamine release from hippocampal slices in the literature, although the possibility deserves investigation. It was hypothesised that the role of dopamine in L-LTP was related to the triggering of protein synthesis that is important for establishing L-LTP. When a tetanus was delivered to S2 in the presence of SCH23390, although protein synthesis may have been blocked, the prior tetanus

to S1 would have triggered the synthesis of ‘plasticity-proteins’ which when distributed throughout the neuron would be captured by tags set on S2 leading to L-LTP on this pathway.

However, it is possible that dopaminergic activation is involved in the setting of tags and not just in triggering of protein synthesis. Experiments by Frey et al., (1993) and, Huang and Kandel, (1995) revealed that dopaminergic agonists induce a slow-onset potentiation that is occluded by three repeated high frequency trains and blocked by protein synthesis inhibitors. Otmakhova and Lisman, (1996) found that D1/D5 agonists increased the magnitude of E-LTP in hippocampal slices. If dopamine is involved in tag setting, then these tags may be susceptible to disruption following the tetanus, if dopaminergic antagonists are present at this time. This interpretation still relies on activation of dopamine receptors post-tetanus.

Nevertheless, the functional consequence of dopaminergic modulation of hippocampal synaptic plasticity is poorly understood. Frey et al., (1990) found that endogenous dopamine is released in the hippocampal slice during tetanisation. This might suggest that dopamine exerts an effect on LTP during the simultaneous activation of NMDA receptors by glutamate release during high frequency stimulation, although this is not entirely clear. There are a number of possibilities. First, dopamine might affect LTP even though it arrives after the tetanus. This may be possible via the suggested role that dopamine has triggering protein synthesis. Behavioural experiments have shown that dopaminergic activity is important hours after training; intrahippocampal dopamine agonists given after training improve memory and antagonists decrease it (Grecksch and Matthies, 1982; Bernabeu et al., 1997). Alternatively, as the experiments of Otmakhova and Lisman, (1998) demonstrate, activation of D1/D5 receptors inhibit depotentiation of previously induced LTP. Therefore, activation of dopamine receptors in by a high frequency tetanus, within a time-window when recently potentiated synapses are susceptible to depotentiation, may render the synapses resistant to processes involved in depotentiation and returning synaptic strength to a ‘resting’ state. This could provide a mechanism for ‘tagging’ recently activated synapses. However, these are speculative suggestions, and future work is necessary in order to understand how dopamine modifies the rules of activity-dependent synaptic plasticity, given the variety of effects that this neuromodulatory pathway has been shown to have both *in vitro* and *in vivo*.

Chapter 7. Dopamine and long-term memory.

7.1 Introduction.

The experiments reported in this chapter address whether or not intrahippocampal infusions of the dopamine D1 receptor antagonist SCH23390 produce delay-dependent impairments in a novel version of the watermaze task called the delayed matching-to-place task (Steele and Morris, 1999).

These experiments were necessary as the first step in a series of future ‘behavioural tagging’ experiments. It is possible that as for L-LTP, the persistence of hippocampal dependent long-term memories can be modulated by non-glutamatergic inputs to the hippocampus. ‘Weak’ memories may be heterosynaptically modulated by non-glutamatergic input to the hippocampus in such a way that a ‘normally’ short-lasting memory may under certain circumstances become more enduring due to activation of neuromodulatory inputs that are important in triggering the distribution of ‘plasticity-proteins’ involved in the long-term storage of information. This is probably an oversimplification, and the situation *in vivo* is likely to be far more complex than that in a hippocampal slice. However, if it is possible to demonstrate that neuromodulatory pathways, such as the dopaminergic system, are involved in the long-term storage of information in certain tasks then it may be possible to manipulate this system in such a way that activation of these pathways results in paradoxical long-term storage that would not occur under ‘normal’ circumstances. Stimulation of the ventral tegmental area (VTA), the primary dopaminergic afferent to the hippocampus (Swanson, 1982), may be one such strategy.

7.1.1 Dopaminergic modulation of memory processes.

There is considerable evidence that suggests that activity of the dopamine system is important in the performance of working memory tasks that are dependent upon the prefrontal cortex. Neurons recorded from the prefrontal cortex of behaving primates (Goldman-Rakic, 1995), and rats (Orlov et al., 1988; Batuev et al., 1990) show sustained firing throughout the brief delay period of a delayed match-to-sample task. Lesions of dopaminergic terminals in the prefrontal cortex disrupts performance on both delayed match-to-sample and delayed non match-to-sample tasks (Brozoski et al., 1979; Bubser and Schmidt, 1990). Administration of high doses of D1, but not D2, antagonists into the prefrontal cortex disrupts performance delayed match-to-sample tasks and decreases the activity of “*delay neurons*” in the prefrontal

cortex (Sawaguchi et al., 1990; Sawaguchi and Goldman-Rakic, 1994; Williams and Goldman-Rakic, 1995). This data clearly indicate a role for dopaminergic modulation of neural processing within the prefrontal cortex during short-term active retention of information i.e., working memory.

Floresco and Phillips, (2001) trained animals in a recognition memory task in a radial maze where animals are required to remember which arm of the maze they last visited to collect a food reward. Good performance in the choice phase is indicated by few entries into an arm that had already been visited previously. Performance is generally good at short delay intervals between the sample and choice phase (30 min) and poor at long-delays (12 hr). On the test day, rats were given an infusion of the D1 receptor agonist SKF81297 into the prefrontal cortex in the interval before retention testing i.e., after the sample phase, and just before the choice phase. The D1 agonist improved subsequent retention when animals were tested at the 12 hr delay, and actually impaired performance at the short 30 min delay. In another study, Didriksen, (1995) used a delayed-non-match-to-position task that also involves the prefrontal cortex. They trained the animals for a number of days on this task and then the animals were given injections of various drugs 30 min before a day of testing. They found that the D1 antagonist SCH23390 produced delay-dependent impairments in performance at long (i.e., 6-9 sec between the sample and test phase) but not short intervals (i.e., 0-3 sec).

7.1.2 Dopaminergic modulation of hippocampal dependent learning and memory.

The role of the dopaminergic system in hippocampal dependent tasks is less well understood. Gasbarri et al., (1996) found that animals given 6-hydroxydopamine lesions of the dorsal and ventral subiculum, and adjacent CA1 fields of the hippocampus were relatively normal in acquisition of the reference memory version of the watermaze, but were impaired in the probe test. However, the performance of the sham operated animals on the probe test was quite poor in this experiment. Experiments have also been conducted on dopamine D1 receptor deficient mice (El Ghundi et al., 1999). These animals did not acquire the reference memory watermaze task as well as controls, nor did they show any preference for the training quadrant in the probe test.

More convincing evidence that dopamine modulates the storage of memory in hippocampal dependent tasks, comes from experiments using inhibitory avoidance paradigms. Bernabeu et al., (1997) found that intrahippocampal infusions of SCH23390 resulted in complete lack of memory for a step down inhibitory avoidance task when the injections were given either 3 or 6 hr after training (i.e., a post-training infusion prior to retention testing), but not 0 or 9 hr. On

the other hand, the dopamine agonist SKF38393 facilitated memory when given 3 or 6 hr, but not 0 or 9 hr post-training (similar effects were seen with activators of the cAMP pathway). In association with learning, these effects were correlated with an increase in binding of D1/D5 receptors in the hippocampus, and increased phosphorylation of CREB at 3 hr post-training. Subsequent studies have established that these effects of dopamine on the cAMP/protein kinase A pathway are hippocampal, and not amygdala dependent (Bevilaqua et al., 1997a).

The reason for administering drugs post-training, as is the case in many of these studies just discussed, is to avoid complications arising from possible effects that such agents may have on sensory processes, and on the initial acquisition and encoding of the task. The supposition is that the drug interferes with processes required for retention of the information, such that, when the animal is re-tested, poor performance reflects the negative effects the drug exerts on retention but not encoding. However, if one can demonstrate that the drug of interest has no effects on initial acquisition, then pre-training treatments may still allow us to differentiate between effects on short and long-term memory. The delayed matching-to-place task has been shown to provide such possibilities (Steele and Morris, 1999).

7.1.3 Delay-dependent impairments of memory.

Delayed-matching-to-sample (DMS) and non-matching to sample (DNMS) tasks are widely used for both rats and monkeys. There is controversy about whether or not these tasks are hippocampal dependent, and this dependency seems to be related to the type of DMS task used and the training protocol (Hampson et al., 1999). There is strong evidence that, when the task involves a spatial component, the hippocampus is required at all delays (Aggleton et al., 1986; Hampson et al., 1999; Steele and Morris, 1999).

In many primate DMS or DNMS tasks, performance is characterised by sustained firing of “*delay neurons*” during the interval between ‘sample’ and the ‘choice’ phase of the task (Goldman-Rakic, 1996). These tasks are generally considered to be synonymous with recognition and working-memory tasks and are dependent upon activity within the prefrontal cortex (Goldman-Rakic, 1995). However, the delayed-match-to-place (DMP) watermaze task cannot be easily compared with a recognition memory task. Although necessary, it is not sufficient for the rat only to recognise the place where the hidden platform is located in the pool, it must also navigate to the correct place. It is this requirement that necessitates the integrity of the hippocampal formation (Steele and Morris, 1999).

The DMP watermaze task is very sensitive to both hippocampal lesions and intrahippocampal infusions of the NMDA receptor antagonist AP5 (Steele and Morris, 1999). In this task, animals are typically trained for four trials per day to learn the position of a hidden platform, the location of which varies from day to day, but remains fixed for all four trials of a given day. After relatively few days training (approximately 5 days of pre-training appears to be sufficient), the performance of this task is characterised by long escape latencies on trial 1 of any given day (i.e., the time taken from the point the animal is placed in the pool to when it finds the platform), but much shorter latencies thereafter. One great advantage of this task over the ‘reference memory’ watermaze task (Morris et al., 1982) is that by varying the delay between trials 1 and 2, one can differentiate between short-term and long-term memory processes as a result of various pharmacological or genetic manipulations (Zeng et al., 2001).

Another advantage of this task is that it allows for the possibility of using a within-subjects experimental design in which the rat is sometimes treated with drug (or vehicle) and vice versa. By pretraining all animals, it is possible to determine whether similar levels of performance are obtained at different trial 1 to trial 2 inter-trial intervals (ITI's) before animals are subject to a given manipulation. Therefore, when it comes to testing the animals with various drugs, we can reasonably assume that they all acquired the basic procedural elements of the task equally. The within-subjects design allows each animal to be tested after infusion of the drug or vehicle, at different trial 1 to trial 2 delays over a period of days, thus affording the possibility to differentiate between short-term and long-term effects on performance.

The effects of intrahippocampal infusions of the dopamine D1 receptor antagonist SCH23390 were assessed in rats trained in the DMP watermaze task. To address the possible delay-dependent effects of this drug, animals were trained in the task with either a short trial 1 to trial 2 ITI (20 min) or a long trial 1 to trial 2 ITI (6 hr).

7.2 Methods.

7.2.1. *The delayed-match-to-place watermaze task.*

The basic procedure for the delayed-match-to-place (DMP) watermaze task, and details of the surgical procedures carried out, were described in Chapter 2 (see Figure 2.4). Rats (adult male Lister Hooded) underwent surgical implantations of bilateral intrahippocampal guide cannulae at least one week prior to pretraining in the DMP task. All rats ($n = 24$) were given 8 days of pretraining with 4 trials per day. Trials began at north, south, east or west, in a pseudorandom sequence, with rats facing the side-walls. During pretraining, the hidden platform was located, on successive days, in one of eight possible locations within the pool (see Figure 7.1). The platform locations were changed between days according to a pseudo-random schedule. Each rat was randomly assigned to one of two, counterbalanced platform location sequences. All trials ended with 30 sec allowed on the platform. The inter-trial interval (ITI) between trials 1 and 2 was either 20 min or 6 hr, with 4 days of each ITI during the 8 days of pretraining. The ITI between trials 2 and 3, and between trials 3 and 4 was always 30 sec.

For preparation of SCH23390, 16 mg of powdered drug was dissolved in 3.2 ml of sterile, millipore filtered aCSF to a concentration of 5 mg/ml (5 $\mu\text{g}/\mu\text{l}$). This solution was vortexed and gently sonicated before being distributed into eppendorfs in 500 μl volumes each and then stored at -20°C .

On each day of DMP testing, animals were infused bilaterally into each hippocampus with (1 $\mu\text{l}/\text{side}$), of either SCH23390 or aCSF, at a flow rate of 0.2 $\mu\text{l}/\text{min}$ over five minutes. During infusion, the animal was free from any restraint. The injection cannulae were left in the guide cannulae for two minutes after infusion to avoid backflow. After injection the dummy cannulae were placed back in the guide cannulae. Behavioural testing began 15 min after the infusion.

Following pre-training, animals were tested over 8 consecutive days, with 4 trials per day in the DMP task the same way as described for pretraining. The hidden platform was located, on successive days, in one of eight possible locations within the pool, and these differed from the pre-training locations. The inter-trial interval (ITI) between trials 1 and 2 was either 20 min or 6 hr. Animals received 4 infusions of aCSF and 4 infusions of SCH23390, two prior to the 20

min ITI and two prior to the 6 hr ITI. The different ITI's varied within subjects in a counterbalanced manner.

Following behavioural testing, all animals were perfused with 0.9 % saline followed by saline buffered formalin. Their brains were removed, placed in formalin, and 20 μm sections were cut for staining. These sections were stained using cresyl violet, and examined under a light microscope. I am grateful to Miss. Jane Knox for performing the histology. The maximal and minimal position of the cannulae at selected coronal planes through the hippocampus was later reconstructed. Figure 7.2 shows that the cannulae were correctly positioned within the hippocampus, towards the ventral pole of the hippocampus, where the majority of dopaminergic fibres are located (Verney et al., 1985).

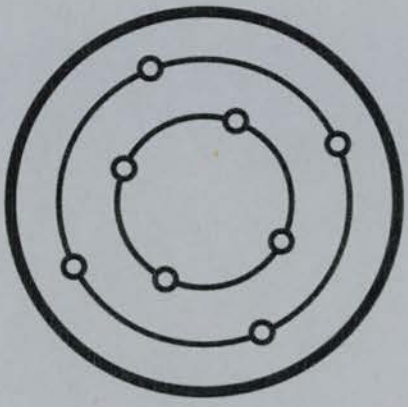
Figure 7.1 The experimental design.

The platform positions used during pretraining and testing are shown. Eight different locations were used, and the positions were different for the pretraining and testing phase of the experiment as shown.

All animals initially underwent surgical implantation of intrahippocampal cannulae and were given at least 7 days to recover. Following this, 8 days of pretraining occurred with both 20 min and 6 hr ITI's between trials 1 and 2. A subsequent 8 days of testing followed where the animals were given either an infusion of aCSF or SCH23390, 15 min before being placed in the pool for trial 1.

EXPERIMENTAL DESIGN

a. Pretraining platform locations.



a. Testing platform locations.

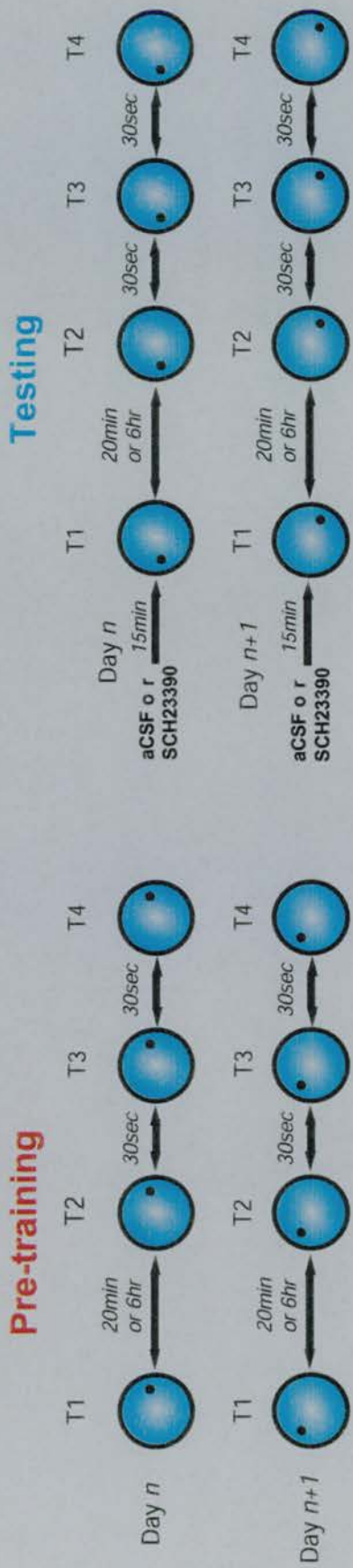
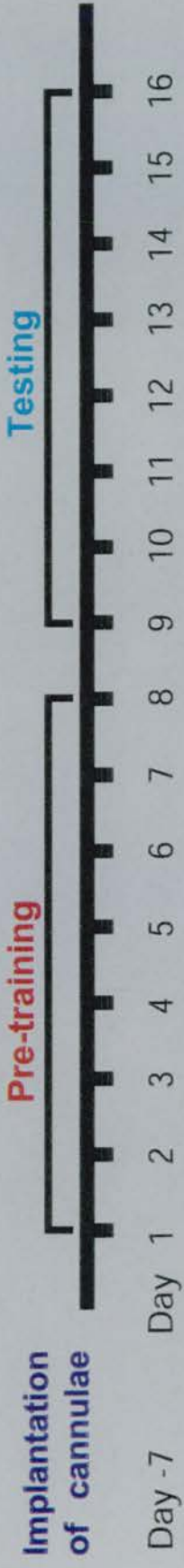
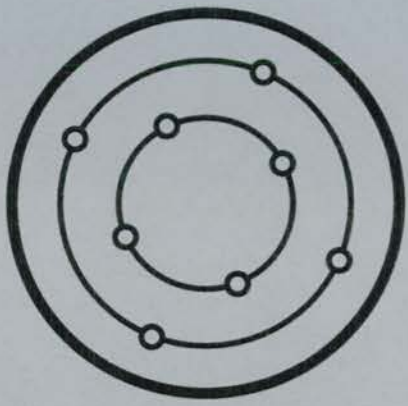


Figure 7.2 Histologically identified bilateral cannula locations for intrahippocampal infusions.

The locations of the cannulae tips as verified from histological sections are represented by the red dot on each plate. The bi-lateral injection sites are predominantly located within the dentate gyrus. However, using the same injection coordinates in the same laboratory, (Riedel et al., 1999) demonstrated that infusions of the AMPA/kainate receptor antagonist LY326325 blocked synaptic transmission in both the dentate gyrus and CA1 indicating that the drug reached all areas of the hippocampus from these injection coordinates.

7.4 Results.

7.4.1 Normal acquisition of the DMP watermaze task during pre-training.

A number of measures of performance were analysed during both pretraining and testing, including (1) Escape latency to find the hidden platform on trials 1 to 4. (2) “Savings” in escape latency between trials 1 and 2 i.e., trial 1 escape latency minus trial 2 latency. (3) Swim speeds on trials 1 to 4. (4) Path length to find the hidden platform on trials 1 to 4. (5) “Savings” in path lengths between trials 1 and 2.

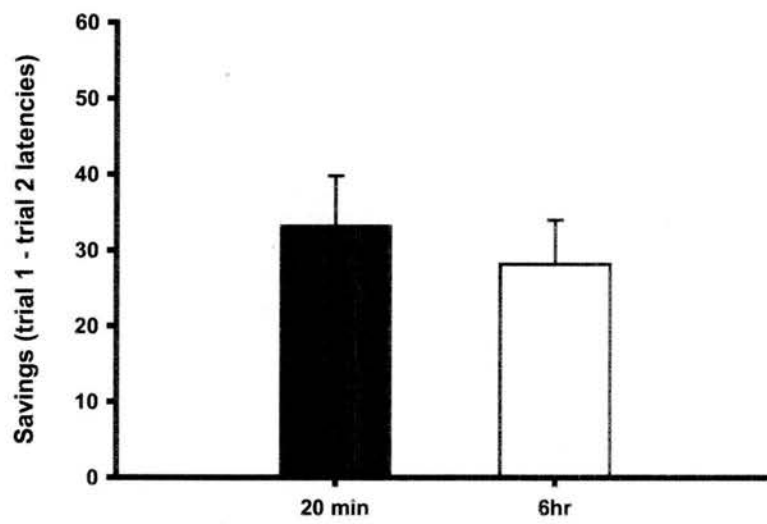
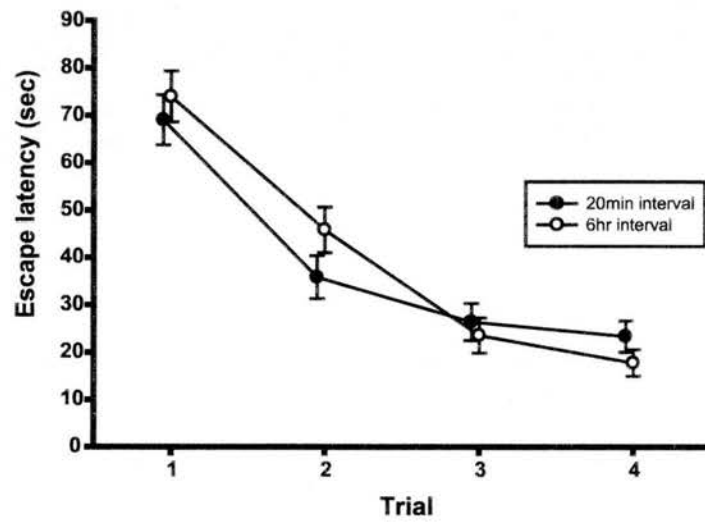
The general pattern of behaviour during pretraining in this task was as follows. Trial 1 was characterised by high escape latencies indicating that the animal has no prior knowledge on the day's platform position (see Figure 7.3). Animals clearly remember the platform position very well, as when placed back in the pool for trial 2, the escape latencies are substantially reduced. This ability to recall the platform position on trial 2 occurred when there was either a 20 min or a 6 hr interval between trials 1 and 2.

The data in Figure 7.3 a, shows escape latencies averaged over the 8 days of pretraining as a function of trials within each day for both the 20 min and the 6 hr inter - trial interval (ITI). During pretraining, with a 20 min ITI, the mean escape latency declined from 68.9 ± 5.3 s on trial 1 to 35.8 ± 4.5 s on trial 2, a saving of 33.1 s. For the 6 hr ITI the mean escape latency declined from 73.9 ± 5.4 s on trial 1 to 45.8 s on trial 2, a saving of 28.1 s. An analysis of variance showed that all animals, whether they had a 20 min or a 6 hr ITI between trials 1 and 2, showed significant improvements in performance across the four trials i.e., a significant main effect of trial [$F(3, 189) = 64.6, p < 0.005$]. There was no significant difference between the 20 min condition and the 6 hr condition across trials 1 to 4 i.e., no interaction between trial and ITI [$F(3, 189) = 2.1, p > 0.05$].

The key feature of the DMP task is the performance on trial 2 following different ITI's. Therefore, analysis of the savings in escape latency between trials 1 and 2 as a function of ITI (20 min or 6 hr) is a key measure of performance in this task. Figure 7.3 b, shows the savings scores for both ITI's during pretraining. There was no significant difference between the 20 min and the 6 hr ITI conditions in savings [$F < 1$], indicating similar levels of acquisition of this task with either a 20 min or a 6 hr between trials 1 and 2.

Figure 7.3 Normal acquisition of the DMP watermaze task during pretraining.

- a. Escape latencies averaged across the eight days of pretraining as a function of ITI between trials 1 and 2.
- b. Savings in escape latency between trials 1 and 2 during pretraining. There were equivalent savings of escape latency across both ITI's, with a trend towards less savings at 6 hr.



7.4.2 Delay-dependent memory deficits at a 6hr, but not a 20 min interval, following intrahippocampal infusions of SCH23390.

Following pretraining, the animals were tested in the DMP task in an identical manner to pretraining with the exception that they received an intrahippocampal infusion of either aCSF or SCH23390 15 min before trial 1 of each day. Representative swim paths on trials 1 to 4 after an infusion of either aCSF or SCH23390, are shown in Figure 7.4 a (20 min ITI) and in Figure 7.4 b (6 hr ITI).

The key finding during testing was that with a 20 min interval, the animals had good memory on trial 2 for the location of the hidden platform following an infusion of either aCSF or SCH23390. However, with a 6 hr interval, although the animals given an infusion of aCSF also had good memory for the platform location, those treated with SCH23390 no longer remembered the location of the platform when tested on trial 2.

Figure 7.5 a, shows escape latencies across trials averaged across the four days where either aCSF or SCH23390 was given before animals were tested with a 20 min trial 1 – 2 ITI. For these groups, the mean escape latency declined from 56.0 ± 5.7 s on trial 1, to 28.7 ± 4.3 s on trial 2, a saving of 27.3 ± 7.3 s. With a 20 min ITI when animals were given an infusion of SCH23390, the mean escape latency declined from 78.7 ± 6.3 s on trial 1, to 41.7 ± 5.2 s on trial 2, a saving of 36.9 ± 7.9 s.

Figure 7.5 b, shows escape latencies across trials averaged across the four days where either aCSF or SCH23390 was given before animals were tested with a 6 hr trial 1 – 2 ITI. For these groups, the mean escape latency declined from 62.0 ± 5.8 s on trial 1, to 30.3 ± 3.7 s on trial 2, a savings of 27.3 ± 7.3 s. With a 6hr ITI when animals were given an infusion of SCH23390, the mean escape latency declined from 66.4 ± 6.4 s on trial 1, to 57.3 ± 5.3 s on trial 2, a savings of only 9.1 ± 8.2 s.

An overall analysis of variance revealed a significant effect of treatment (aCSF or SCH23390) [$F(1, 42) = 21.7, p < 0.0005$], a significant interaction between treatment and trial [$F(3, 132) = 4.7, p < 0.005$] and also a significant interaction between treatment, trial and ITI [$F(3, 132) = 3.2, p < 0.05$]. Because this triple interaction was found we decided to focus on the savings between trials 1 and 2, as the interaction is likely to result from differences between these two trials.

Savings between trials 1 and 2 clearly highlights that the effect that SCH23390 had on performance in this task was delay-dependent (Figure 7.5 c). The savings between trials 1 and 2 at the 20 min ITI did not differ significantly between the aCSF and the SCH23390 animals (27.3 ± 7.3 for the aCSF animals, and 36.9 ± 7.9 for the SCH23390 group). However, there is a clear difference between groups at the longer 6 hr ITI (31.7 ± 5.8 for the aCSF animals, and 9.1 ± 8.2 for the SCH23390 group). Subsequent performance of the SCH23390, 6 hr ITI group did improve on trials 3 and 4 indicating that the animals remembered the position after eventually finding it on trial 2. This is confirmed by a statistical significant interaction between treatment and ITI [$F(1, 42) = 5.6, p < 0.05$].

Figure 7.4 Swim paths during DMP testing.

- a. Representative swim paths on trials 1 and 2 with a 20 min ITI for animals given an infusion of aCSF or SCH23390. Note that the animals show circuitous paths on trial 1 when the platform location is unknown. Both the aCSF and SCH23390 rats take shorter paths to the platform on trial 2 after a 20 min interval between trials.
- b. Representative swim paths on trials 1 and 2 with a 6 hr ITI for animals given an infusion of aCSF or SCH23390. Only the aCSF animals show a relatively direct path to the platform on trial 2, whereas the SCH23390 rats show no retention of the platform position.

20min aCSF



Trial1

20 min ITI



Trial 2

30 sec ITI



Trial 3

30 sec ITI



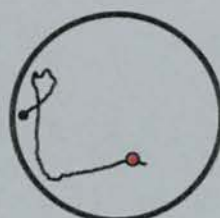
Trial 4

20min SCH23390



Trial1

20 min ITI



Trial 2

30 sec ITI



Trial 3

30 sec ITI



Trial 4

6hr aCSF



Trial1

6 hr ITI



Trial 2

30 sec ITI



Trial 3

30 sec ITI



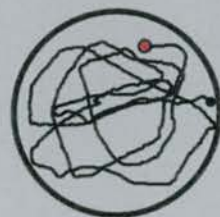
Trial 4

6hr SCH23390



Trial1

6 hr ITI



Trial 2

30 sec ITI



Trial 3

30 sec ITI

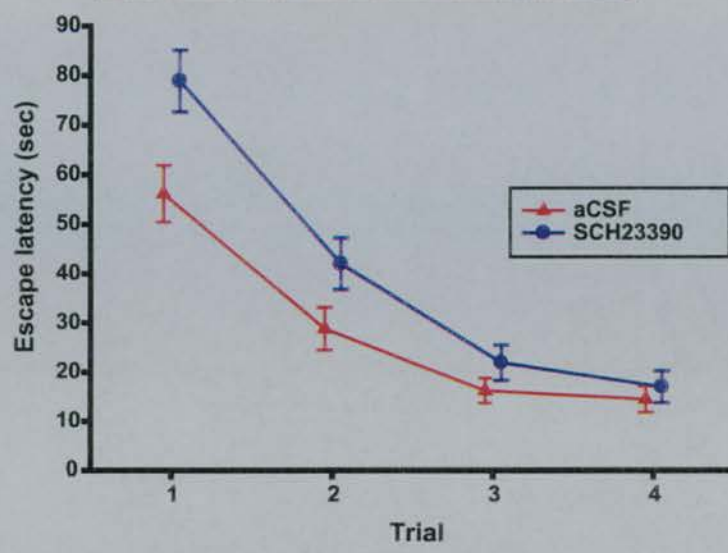


Trial 4

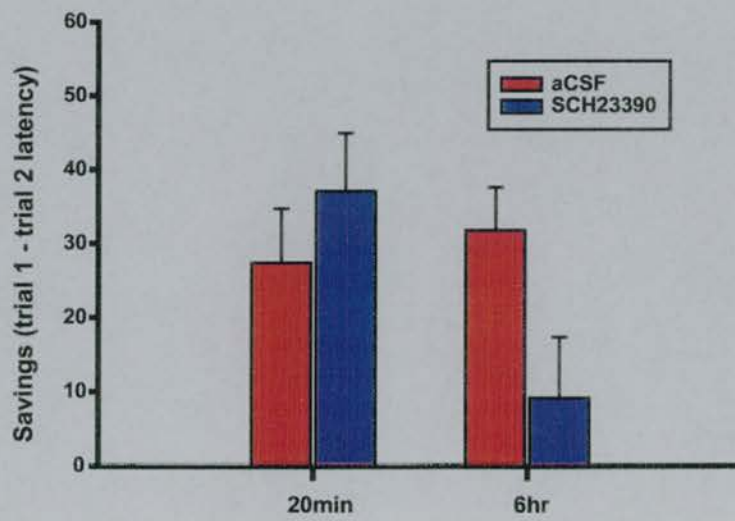
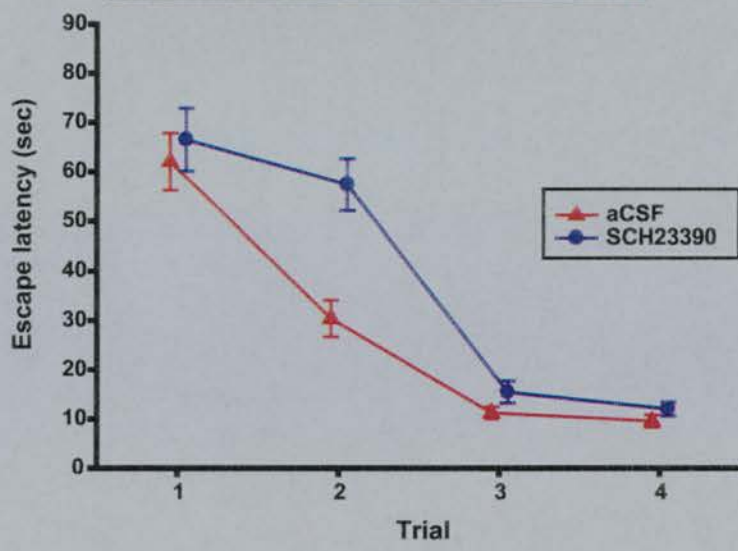
Figure 7.5 Intrahippocampal infusion of SCH23390 results in a delay-dependent impairment in performance at long, but not short, retention intervals.

- a. Escape latencies across trials averaged across the four days where either aCSF or drug was given before animals were tested with a 20 min trial 1 – 2 ITI.
- b. Escape latencies across trials averaged across the four days where either aCSF or drug was given before animals were tested with a 6 hr trial 1 – 2 ITI.
- c. Savings in escape latency between trials 1 and 2 for animals treated with aCSF and SCH23390. With a 20 min ITI, there is no significant difference between animals trained with either aCSF or SCH23390. However, with a 6hr delay, animals given an infusion of SCH23390 prior to trial 1 show significantly less savings on trial 2.

20min interval between Trials 1 and 2.



6hr interval between Trials 1 and 2.



7.4.3 Are there delay independent effects of intrahippocampal infusions?

Occasionally, sensorimotor abnormalities in rats treated with SCH23390 did occur. When side effects occurred following infusions with SCH23390, they appeared predominantly on trial 1 with little effect on subsequent trials. Hypoactivity and 'sluggishness' were the side effects apparent in the SCH23390 treated animals. This resulted in slower swim speeds on trial 1 of some days; however the effects were mild and acute. An analysis of variance of swim speeds revealed a significant interaction between treatment (aCSF or SCH23390) and trial [$F(3, 135) = 6.1, p < 0.05$] see Figure 7.6.

Paired sample t-test comparisons (with Bonferroni correction for multiple comparisons) showed that swim speeds were significantly different between the aCSF and SCH23390 groups with a 20 min ITI on trial 1 (Mean speed for aCSF = 0.27 ms^{-1} , SCH23390 = 0.22 ms^{-1} , $t = 5.1, p < 0.005$). Swim speeds did recover in both groups and there were no significant differences on trial 2 between these two groups (Mean speed for aCSF = 0.29 ms^{-1} , SCH23390 = 0.27 ms^{-1} , $t = 2.3, p > 0.05$). Similar results were found for the 6 hr ITI groups as swim speeds were significantly different between the aCSF and SCH23390 groups on trial 1 (Mean speed for aCSF = 0.27 ms^{-1} , SCH23390 = 0.23 ms^{-1} , $t = 4.7, p < 0.005$) but had recovered by trial 2 (Mean speed for aCSF = 0.30 ms^{-1} , SCH23390 = 0.30 ms^{-1} , $t = 0.1, p > 0.05$).

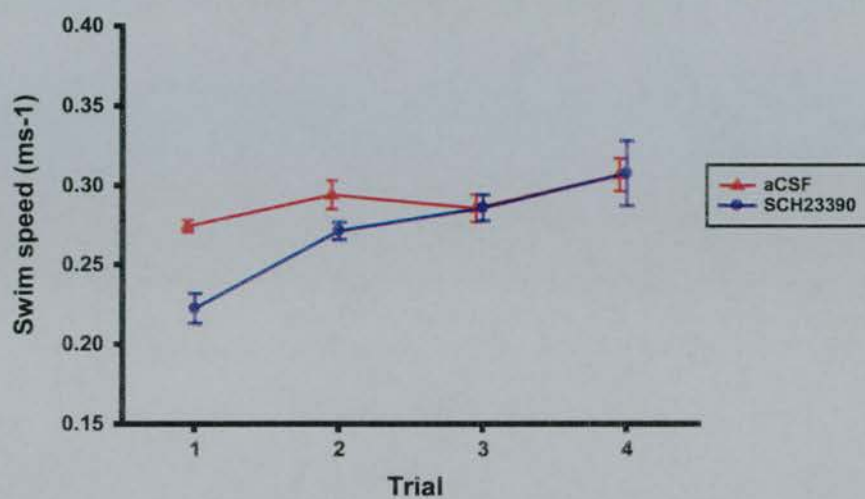
As there were some delay-independent effects of infusions on trial 1 performance, analysis of path length to find the platform was conducted. Differences in path lengths may provide a more sensitive measure of performance given the effect of infusions on swim speeds. This effect may account for at least some of the differences in escape latency to find the platform.

Figure 7.7 a, shows path lengths across trials averaged across the four days where either aCSF or drug was given before animals were tested with a 20 min trial 1 – 2 ITI. For these animals, the mean path length declined from $14.6 \pm 1.6 \text{ m}$ on trial 1, to $7.7 \pm 1.2 \text{ m}$ on trial 2, a saving of $6.9 \pm 2.1 \text{ m}$. With a 20 min ITI when animals were given an infusion of SCH23390, the mean path length declined from $15.8 \pm 1.3 \text{ m}$ on trial 1, to $11.4 \pm 1.4 \text{ m}$ on trial 2, a saving of $4.3 \pm 1.8 \text{ m}$.

Figure 7.6 Slower swim speeds on trial 1 after infusions of SCH23390 but not aCSF.

- a. Swim speeds across trials averaged across the four days where either aCSF or SCH23390 was given before animals were tested with a 20 min trial 1 – 2 ITI.
- b. Swim speeds across trials averaged across the four days where either aCSF or SCH23390 was given before animals were tested with a 6 hr trial 1 – 2 ITI.

20min interval between Trials 1 and 2.



6hr interval between Trials 1 and 2.

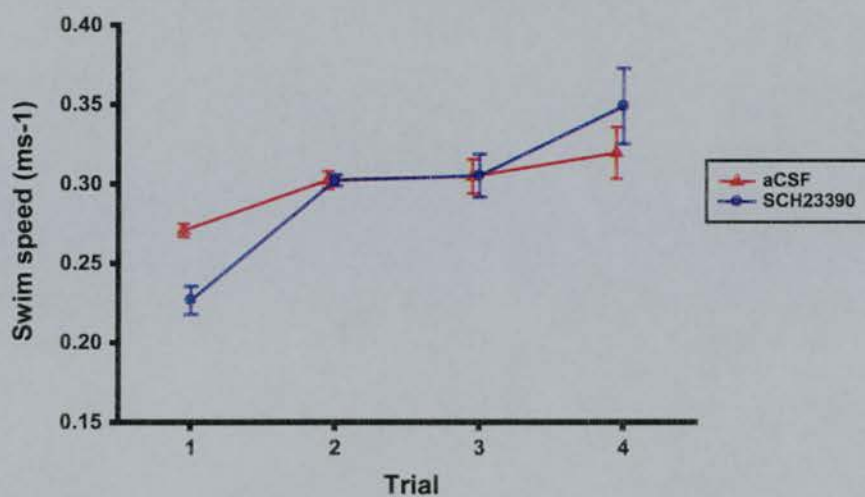


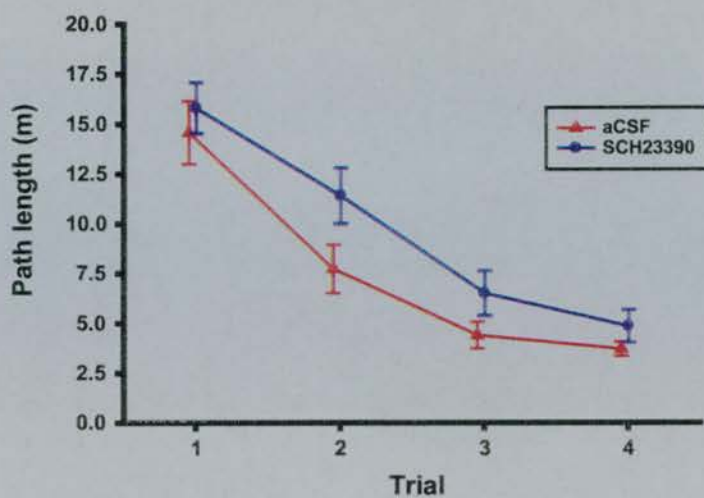
Figure 7.7 b, shows path lengths across trials averaged across the four days where either aCSF or drug was given before animals were tested with a 6 hr trial 1 – 2 ITI. For these animals, the mean path length declined from 17.5 ± 1.6 m on trial 1, to 10.4 ± 1.3 m on trial 2, a savings of 6.9 ± 2.1 m. With a 6hr ITI when animals were given an infusion of SCH23390, the mean path length increased from 14.6 ± 1.4 m on trial 1, to 17.8 ± 1.6 m on trial 2, a negative savings of -3.2 ± 2.0 m.

An overall analysis of variance revealed a significant effect of treatment (aCSF or SCH23390) [$F(1, 44) = 6.5, p < 0.05$], a significant interaction between treatment and trial [$F(3, 132) = 6.2, p < 0.001$], and a significant interaction between treatment, trial and ITI [$F(3, 132) = 2.8, p < 0.05$]. The delay - dependent effect that SCH23390 had on performance was also found to hold true for path length savings between trials 1 and 2 (Figure 7.7 c). The savings between trials 1 and 2 at the 20 min ITI did not differ significantly between the aCSF and the SCH23390 animals (6.9 ± 2.1 for the aCSF animals, and 4.3 ± 1.8 for the SCH23390 group). However, there is a clear difference between groups at the longer 6 hr ITI (7.2 ± 1.8 for the aCSF animals, and -3.2 ± 2.0 for the SCH23390 group). Subsequent performance of the SCH23390, 6 hr ITI group did improve on trials 3 and 4 indicating that the animals remembered the position after eventually finding it on trial 2. This is confirmed by a statistical significant interaction between treatment and ITI [$F(1, 45) = 5.6, p < 0.05$].

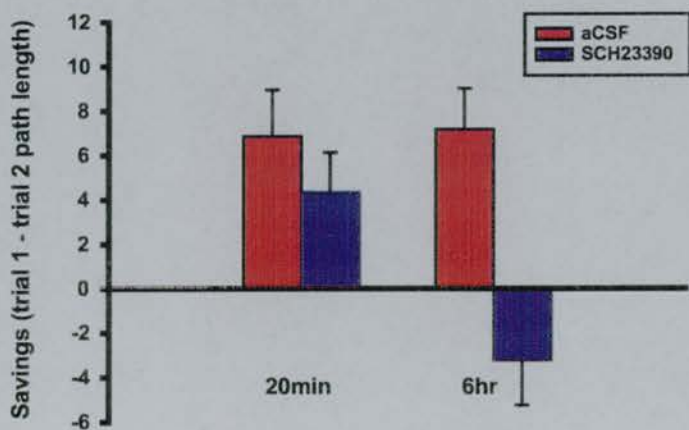
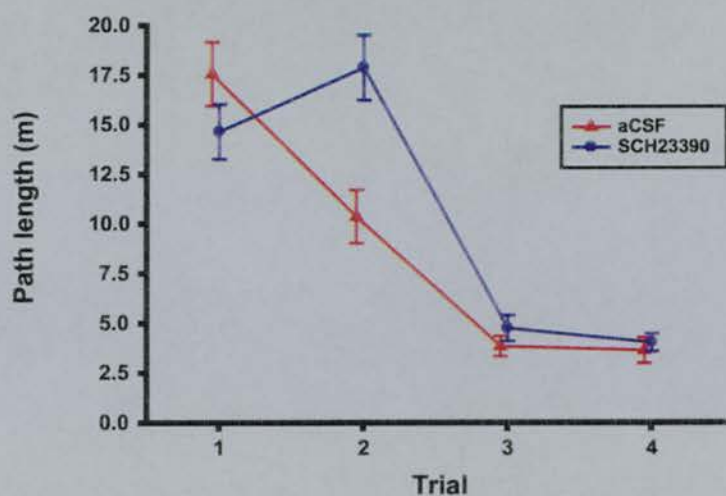
Figure 7.7 The delay-dependent effect of SCH23390 is also evident in the distance animals swim before finding the hidden platform on trial 2.

- a. Escape latencies across trials averaged across the four days where either aCSF or SCH23390 was given before animals were tested with a 20 min trial 1 – 2 ITI.
- b. Escape latencies across trials averaged across the four days where either aCSF or SCH23390 was given before animals were tested with a 6 hr trial 1 – 2 ITI.
- c. Savings in path length between trials 1 and 2 for animals treated with aCSF and SCH23390. With a 20 min ITI, there is no significant difference between animals trained with either aCSF or SCH23390. With a 6hr delay, animals given an infusion of SCH23390 prior to trial 1 show negative savings, i.e., the distance they swim before they find the platform on trial 2 is longer than that taken on trial 1.

20min interval between Trials 1 and 2.



6hr interval between Trials 1 and 2.



7.4 Discussion.

7.4.1 Delay-dependent impairment following the blockade of hippocampal D1 receptors.

The results of these experiments revealed that intrahippocampal infusion of the dopamine D1 receptor antagonist SCH23390 causes a delay-dependent impairment of spatial memory. Short-term retention at 20 min is intact, whereas the animals' memory after 6 hr is significantly impaired. E-LTP is similarly unaffected by application of SCH23390 to hippocampal slices, with L-LTP being completely blocked by this D1 receptor antagonist. This is suggestive evidence for a link between LTP and spatial learning in terms of the role of dopamine in these related processes.

There were some delay-independent impairments associated with infusion of this drug, however these were not present on trial 2 at either the 20 min or 6 hr ITI, therefore they are cannot completely account for the differences observed. The dose of the drug used in the study was quite high. In the present experiments, animals were bilaterally infused with 5 $\mu\text{g}/\mu\text{l}$, whereas previously reported effective doses of the drug in hippocampal dependent tasks are in the range of 0.5 – 2.5 $\mu\text{g}/\mu\text{l}$ (Bernabeu et al., 1997; Bevilaqua et al., 1997b; Bevilaqua et al., 1997a; Izquierdo et al., 1998b; Izquierdo et al., 1998a), whereas Seamans et al., (1998) used a dose of with 5 $\mu\text{g}/\mu\text{l}$ infused into the prefrontal cortex in a spatial working memory task and reported no side effects. However, this drug had not been previously tested for its effects on performance in spatial tasks like the watermaze. It was considered important to first establish an effective dose for achieving an effect on performance without any marked side effects. It may therefore be worth investigating whether similar results are obtained with lower doses of the drug in order to minimise the side effects that sometimes were found.

An additional complication that shall be addressed in future experiments concerns the issue of 'state-dependent' learning. State-dependent learning is a phenomenon in which the retrieval of newly acquired information is possible only if the subject is in the same sensory context and physiological state as during the encoding phase (Overton, 1964). It is possible that the animals trained with the 20 min ITI were still influenced by the drug on trial 2, whereas with a 6 hr ITI there are less chances of the drug being present and so these animals perform the task in a different 'state'. This shall be addressed in future experiments by training a group of animals with a 6 hr ITI but performing the infusion before trial 2 instead of trial 1.

However, these results do point to clear delay-dependent effects of this drug on DMP performance and stand as the first such demonstration in a hippocampal dependent task with pre-training drug treatments. Previously, post-training infusions of this drug had shown effects on short-term but not long term retention, but this was with inhibitory avoidance tasks where robust long-lasting retention is achieved with a single training trial (Bernabeu et al., 1997; Bevilaqua et al., 1997b; Bevilaqua et al., 1997a; Izquierdo et al., 1998b; Izquierdo et al., 1998a). The advantage of the present study is that we can clearly differentiate between short and long-term retention repeatedly over successive days. This is analogous to the effect that SCH23390 has on LTP in the hippocampal slice, i.e., no effect on E-LTP, with a clear inhibitory effect on L-LTP.

7.4.2 Behavioural tagging.

These experiments were conducted as a first step in the development of ‘behavioural tagging’ experiments. This ‘literal’ application of the synaptic tag hypothesis to the behaving animal may be an oversimplification, but it would be interesting to establish whether synergistic interactions occur between tasks that trigger persistent memories (‘strong’ training) and those associated with rapidly decaying memories (‘weak’ training). That is, if a task inducing a weak memory occurs just after one inducing a strong memory, might the former be remembered for longer? Following from this it may be possible to explore whether long-lasting memories could be induced if protein synthesis was ‘primed’ by prior behavioural/pharmacological or even electrophysiological (e.g. stimulation of the ventral tegmental area) activation. The major challenge to this kind of experiment is to find a task whereby a common pool of neurons are activated by both training regimes in order to allow for the possibility of heterosynaptic interactions and the distribution of ‘plasticity-proteins’ to the same cells, as in the slice experiments. This may be possible by (a) conducting both tasks in the same spatial context, taking care to minimise proactive and retroactive interference (e.g. DMP task in open watermaze vs. radial maze insert); and (b) Making partial lesions of the hippocampus such that only 25% of the hippocampus is left intact thereby enhancing the probability that the two tasks would use an overlapping pool of neurons. Further experiments could involve the use of intra-hippocampal infusions of SCH233900 to explore whether long-lasting memories could be induced if protein synthesis was primed by prior behavioural (or pharmacological) activation.

Chapter 8. General Discussion.

8.1.1 Dissociation of E-LTP from L-LTP.

The experiments reported in chapters 3 and 4 showed that E-LTP and L-LTP can be dissociated according to the tetanic stimulation used to induce either form of potentiation. It is widely held that the main difference between the induction of E-LTP and L-LTP is that repeated tetanisation with high frequency trains separated temporally is necessary in order to see the sustained potentiation at 8-10 hr post-tetanus that characterises true L-LTP, whereas E-LTP does not have this requirement.

The potentiation induced by repeated tetanisation with high frequency trains was found to be reduced by the application of emetine, although the effect was incomplete. The experiments conducted to measure newly translated proteins with [³⁵S]-methionine indicate that one possibility is that longer applications of emetine are needed in order to completely block translation at least in the recording chamber used. However, inhibiting protein synthesis with emetine clearly reduced the magnitude of LTP induced with multiple high frequency trains indicating that this persistent form of LTP involved protein synthesis.

An important issue to raise relates to the potential dissociation between the level of potentiation induced initially and its persistence over time. Viewed from the perspective of a single synapse, the dissociation seems clear – potentiation may either be stable or decay to baseline. Clearly it would be desirable to establish such a dissociation in field recordings also. Comparison of the potentiation induced by 11 and 21 pulses reveals only a partial dissociation. After 10 min, potentiation was 129 % and 141 % following the 11 and 21 pulse induction protocol respectively. At 360 min, this had declined to 95 % and 129 % respectively, reflecting declines of 34 % and 12 % respectively. This pattern is equally consistent with the idea of some threshold of short-term potentiation having to be crossed to observe persistent LTP, as with the alternative idea that the magnitude of initial potentiation is independent of the duration for which potentiation may last. A way to investigate this potential dissociation further was to find ways to induce L-LTP without merely increasing the afferent glutamatergic activation at the time of induction, i.e. heterosynaptic activation either before or after the inducing stimulus.

8.1.2 The mechanisms of heterosynaptic modulation of L-LTP and long-term memory.

The key findings of the experiments that focussed on the heterosynaptic modulation of LTP and memory persistence were as follows (1) both a ‘strong before weak’ and a ‘weak before strong’ protocol can result in L-LTP on the weakly tetanised input; (2) The ability of ‘strong’ stimulation to rescue this LTP on the weakly tetanised input is dependent upon activation of the NMDA receptor during the ‘strong’ tetanus; (3) Synergistic activity between both NMDA and dopaminergic receptors may be required to induce persistent L-LTP, with the activation of dopamine receptors being important both during and after the tetanic stimulus; and (4) dopaminergic input to the hippocampus is important in the storage of long-term but not short-term retention of information in a spatial learning task.

The first of these experiments provide a replication of some of the original work of Frey and Morris, (1997, 1998a). These experiments point to the fact that the persistence of LTP is not just determined at the time of LTP induction but is critically influenced by events occurring both prior and subsequent to the induction of LTP at any individual population of synapses. Interactions between ‘local’ synaptic tags and ‘diffusely’ synthesised proteins govern the persistence of LTP. If heterosynaptic synthesis of ‘plasticity-proteins’ occurs long before the setting of tags, no interactions can occur. If the time window between these two events is small (as in the ‘Strong before weak’ experiments) this results in the optimal availability of ‘plasticity-proteins’ which can subsequently interact with the setting of synaptic tags on an independent pathway. If tags are set before the distribution of proteins (as in the ‘weak before strong’ experiments) then as long as the interval between these events is within the time in which the tags are active, interactions also take place. What this allows for though is a more dynamic regulation of the persistence of LTP. The synthesis and distribution of ‘plasticity-proteins’ need not be locked to discrete patterns of synaptic activity, although this may occur under certain circumstances. In a system such as the hippocampal slice these events may be discretely locked to events like tetanic stimulation. However, the situation is likely to be more dynamic *in vivo*. This point is addressed again below in relation to the behavioural experiments with SCH23390.

The second key finding relates to the conditions necessary in order to observe the heterosynaptic rescue of decremental LTP induced at weakly tetanised inputs. Blocking the NMDA receptor was found to prevent such interactions from taking place. Activation of NMDA receptors may be crucial in the synthesis and distribution of proteins captured at tagged synapses. Identifying the conditions in which such interactions occur, and

investigating whether the induction of LTP can be dissociated from the synthesis of ‘plasticity-proteins’ would be worthwhile. This is addressed further below.

The third key finding is that blocking dopamine D1 receptors either during or after ‘strong’ tetanic stimulation inhibits the persistence of LTP for > 2 hr. These experiments were undertaken in order to determine whether dopamine receptor-dependent L-LTP would be induced even during the blockade of these receptors at the time of LTP induction. Clearly, by blocking D1 receptors L-LTP is inhibited suggesting that synergistic activity of both NMDA and D1 receptors is important in generating persistent L-LTP. The precise role of dopamine in LTP has yet to be elucidated, and further work would be required to characterise its role. It was hypothesised that dopamine receptors play a key role in the synthesis of the ‘plasticity-proteins’ necessary for L-LTP although this is not known for certain.

The fourth key finding involved extrapolating to the situation *in vivo*, if an animal is performing a task that is emotionally arousing (a situation known to induce persistent long-term memory, see Cahill and McGaugh, 1998) then the proteins produced may be used to strengthen the traces encoded at the activated synapses. Alternatively, if an animal incidentally encodes information before or after an emotionally salient event the information encoded may be retained for a long time. Neuromodulatory inputs to the hippocampal formation may be critical in mediating the effects of emotional arousal via their role in the synthesis of ‘plasticity-proteins’ necessary for long-term storage. It is from this perspective that the behavioural experiments of Chapter 7 provide the basis for future work to investigate these possibilities. The dopaminergic input to the hippocampus is one of a number of neuromodulatory pathways which may be sufficient to generate such heterosynaptic effects *in vivo*.

8.1.3 The future of synaptic tagging.

There are a number of possible solutions to the ‘synapse-to-nucleus’ problem. The synaptic tag hypothesis provides a mechanism for maintaining the input-specificity of LTP even though L-LTP is dependent on translation and transcription that occur in the cell body. However, just as there are multiple mechanisms involved in LTP induction (Sanes and Lichtman, 1999) and memory formation (Martin et al., 2000; Martin and Morris, 2002) there may be multiple mechanisms for generating L-LTP depending on the circumstances and the circuits in which the plasticity is occurring. This point is especially applicable to the following question; what is the synaptic tag? As was discussed in the introductory chapter, there may be many, each of which is required to fulfill four criteria in order to be considered

as likely candidates. Each candidate tag may function under particular circumstances depending on the stimuli used to induce the tags, and on the region of the brain in which they are activated. Some may mediate plasticity over relatively short time periods, whereas others may have a longer role. If synaptic tagging occurs in regions other than the hippocampus it would seem unlikely that one molecule or event could integrate synaptic activity over different spatial and temporal domains. Local protein synthesis as a means of avoiding the need for trafficking of proteins from the nucleus to individual synapses cannot be completely ruled out as contributing to a certain point. However, it does seem likely that the cell body is required to participate at some point. A neuron is an integrative computational unit. In addition to summing excitatory postsynaptic potentials it might also keep a ‘record’ of its own history of activation. By referring to this record, decisions could be made concerning whether the cell produces molecules (such as ‘plasticity-proteins’) that could play a part in the stabilisation of plasticity. The ‘decision’ to utilise these molecules provided by the cell may rest with the individual synapses, but as these sites only possess a limited record of the history of activation, the decision must be shared between the synapses and the nucleus.

A great limitation in these experiments is the simple fact that achieving baseline stability for long periods is extremely difficult. However, a number of interesting future experiments will help elucidate the mechanisms of synaptic tagging further. The experiments reported in Chapters 5 and 6 focused on the conditions in which E-LTP could be ‘transformed’ into a more persistent L-LTP through a heterosynaptic interactions between overlapping inputs to the same CA1 population. One possible line of investigation would be to determine whether or not setting of synaptic tags can be dissociated from the induction of LTP. Preliminary observations of Morris and Frey, (1999) suggest that the setting of synaptic tags is dependent upon activation of the NMDA receptor. Would it be possible to set a tag that is capable of recruiting proteins in a synapse specific manner, if the tag was set either when the induction of LTP was blocked or even independently of the induction of LTP? A ‘strong’ tetanus would be delivered to S1, shortly following this some manipulation to an independent input, S2, would occur such that this manipulation may set tags capable of sequestering proteins that were randomly distributed throughout these overlapping CA1 neurons. The manipulation would have to be specific to this input pathway and be associated with no change in the initial response, but LTP may emerge at later stages. Slow onset potentiation has been reported to occur after application of activators of the cAMP pathway (Frey et al., 1993), dopaminergic agonists (Huang and Kandel, 1995) c.f. (Swanson-Park et al., 1999), and neurotrophins such as BDNF. Although such manipulations are not synapse specific, and in some cases are dependent on protein synthesis (Huang and Kandel, 1995). Therefore, it maybe difficult to dissociate setting of tags from triggering translation. However, if it were possible to

selectively ‘tag’ one input pathway possibly via intracellular injections of drugs, then it may be possible to address these questions. Recent experiments with local application of dopamine agonists suggest that the changes induced by the application are at least specific to the local area of application (Smith et al., 2002).

8.1.3 How late should late-LTP be?

It is tempting to speculate that the mechanisms underlying the persistence of LTP are similar to those underlying the persistence of long-term memory. This clearly is an oversimplification but admittedly provided the background to the experiments looking at the role of dopamine in both L-LTP and long-term memory.

Memories can clearly last a lifetime, but whether LTP needs to be indefinitely persistent is not so clear. Memories may persist for relatively short or long periods depending on the area of the brain subserving their traces. Many people view the hippocampus as a temporary store for memories that ultimately reside within the cortex (Squire, 1992). Thus the persistence of LTP may be determined by the circuit in which the potentiation is induced, different persistence mechanisms may exist that are adapted to the storage requirements for particular cells in particular brain areas. A related conceptual issue relates to the basic attempt to relate an artificial phenomenon such as LTP induced by multiple trains of high frequency trains to a rich phenomenon such as long-term memory. The persistence of L-LTP, as has been shown, is not simply a result of events occurring at the time of induction, i.e., immediately following a single episode. The persistence of memories is also more than likely to be determined by events occurring prior or subsequent to the encoding of the information e.g. the ‘emotional state’ the animal is in, or attentional processes which indirectly influence what features of a salient event are encoded. In addition, persistence may be influenced by off-line processing such as explicit or implicit rehearsal, possibly during sleep (Stickgold et al., 2001).

Synaptic tagging may maintain traces encoded by the hippocampus for long periods so that they are subsequently transferred to the cortex. Alternatively, if the hippocampus is the ‘site’ of storage for certain types of memories then synaptic tagging may determine the variable persistence of certain memories encoded by this structure. The experiments of Frey and Morris, (1997, 1998a) have provided much interest in questions of this sort and given insight into one of the fundamental questions in the field of synaptic plasticity and memory.

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